

A SUMMARY OF THE PROCESSES OF REPRODUCTION WITH
SPECIAL REFERENCE TO A BIOLOGICAL TEST FOR PREGNANCY
IN THE EQUINE.

by

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A SUMMARY OF THE PROCESSES OF REPRODUCTION WITH
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I. PHYSIOLOGY OF REPRODUCTION.

In animals it is usual for the sexual activities to be limited to a particular time or times of the year. This period at which the sexual organs exhibit a special activity is termed by Heape "the sexual season", as distinct from "the breeding season" which he regards as the whole of the time an animal is concerned in the production of young. This includes the sexual season, the period of gestation, parturition and lactation. Most animals, unlike man, are not capable of mating except during this restricted season. Also mating does not occur throughout this period except in rabbits and perhaps in primates, but only at certain regular intervals.


The beginning of the sexual season is marked by the onset of pro-oestrus or the "coming on heat" stage. During this stage the uterus increases in size/

size and becomes congested and in the later stages there may be passage of blood to the exterior. Pro-oestrus is succeeded by oestrus, or the stage of "heat", the only time during the sexual season at which copulation takes place. If the animal is not mated or if conception does not occur the reproductive organs undergo regressive changes during the next stage, metoestrus, and gradually resume their quiescent stage. In the dog, however, a condition known as pseudo-pregnancy follows that of oestrus, in which the changes simulate those of pregnancy, but to a less marked degree. At the end of this stage the organs revert to their quiescent state. Animals which have only one heat period during a sexual season are known as "monoestrus"; in others the metoestrous period is followed by a short quiescent stage known as dioestrus. This in turn gives way to pro-oestrus until the sexual season is over.

If fertilisation should occur during oestrus, the uterus continues to develop, instead of undergoing involution and pregnancy results. Gestation ends with parturition, followed by lactation. The end/

end of lactation marks the end of the breeding season.

In the normal unmated mouse and rat, oestrus occurs about every five days. Allen (3) gives the length of the cycle in the mouse as between four and five days and Parkes (61a) an average of 6.2 days based on over 1,000 cycles. The cycle in the rat according to Long and Evans lasts 5.4 days (52). The mouse becomes mature about the age of seven weeks and the rat at about ten to eleven weeks. The cycle was found to be lengthened 12 days in the mouse and 13 days in the rat following sterile copulation. This is due to the occurrence of pseudo-pregnancy. After parturition, oestrus occurs within 24 hours and is then absent during lactation (61a). Long and Evans (52) state that this period, during which oestrus is absent in the rat, varies with the size of the litter, from 25-40 days. However, increasing the length of lactation does not prolong lactation.



In Britain, the sexual season in the mare normally occurs in the spring and early summer. Some breeds exhibit oestrus earlier, e.g. the thoroughbred, and some later, e.g. the Shetland. During/

During this period there is a succession of dioestrous cycles. According to Marshall (1922) the normal dioestrous cycle lasts about three weeks and the oestrous period a week, but it may vary by three or four days (55a). Seaborn and Champy state that heat occurs at intervals of three to four weeks and lasts for six days (67). They further divide the heat period into two stages, a preliminary one during which the mare shows all the signs of heat but will not accept the horse, and a second one, the stage of acceptance. Seaborn later regarded the oestrous cycle in the mare as lasting 24 days (68). This period he divided into four stages: (1) rest, about 8 days, (2) pro-oestrus, 3 days, (3) oestrus, 3 days and (4) metoestrus, about 10 days. Aitken in 1927, found that the interval between heat periods varied from 20-25 days with an average of 22-23 days(1). The duration of heat was 4-11 days with an average of 7. Whereas Seaborn and Champy found that the heat period was divisible into a preliminary stage of refusal and a second stage of acceptance, Aitken states that most mares will accept the stallion at any time during heat. There is considerable variation in the signs of oestrus shown by the mare and the condition can only be determined/

determined definitely by the behaviour of the mare towards the stallion. As the mares of Seaborn and Champy and Aitken were tested by the stallion for the heat period, it is difficult to understand the marked diversity of opinion on this subject.

After parturition the first heat period in the mare, the "foal" heat, occurs in 11 days, but this time tends to be irregular, and some mares may not come into heat until the 17th day following parturition (55a).

The Ovarian Cycle.

As an animal approaches puberty or the onset of a new sexual season or cycle, one (or more) of the Graafian follicles (according to the size of the litter) undergoes maturation. The follicle increases in size and eventually this causes it to protrude beyond the surface of the ovary. Expulsion of the ovum takes place when the follicle ruptures. The cause of this rupture is usually regarded as increased tension of the follicular fluid.

The time of ovulation varies in different animals. In some, e.g. rabbit, ferret and cat, it is dependent on the act of coitus. In others, ovulation/

ovulation occurs spontaneously during oestrus. This is the case in the rat, mouse, guinea-pig, bitch, sow and cow (55b).

Most of the evidence regarding the time of ovulation is indirect, at least as far as the domesticated animals are concerned. It is stated that the sow can be served most successfully on the second or third day of heat and that service before this time is frequently not followed by pregnancy. The fact that artificial insemination can be used successfully as a method of impregnation is regarded as evidence of ovulation occurring spontaneously during oestrus. This method has been practised successfully in mares, donkeys and cows (55b). Spallanzani in 1874 impregnated a bitch by this method. This has been repeated by many others and as a result of the success of this method Marshall states that "consequently it may be concluded that these animals ovulate independently of coition". While this is probably correct, it does not seem permissible to argue that ovulation occurs spontaneously during oestrus, from the fact that artificial insemination is followed by pregnancy. It/

It might quite well be that the methods adopted in such practices are in themselves sufficient to induce ovulation. In such circumstances it seems that the only satisfactory way to decide the spontaneity of ovulation would be to depend on an examination of the ovaries of animals. This was the method adopted by Seaborn (68) who killed a number of mares at different stages of the oestrous cycle. Rupture of the follicles was noted to occur on the second and third day of oestrus proper, i.e. during the stage of acceptance. Ewart (21a) arrived at a very similar conclusion, namely, that ovulation very often does not occur until near the end of the oestrous period. Aitken examined his mares by rectal palpation and found that it occurred usually during the last or second last day of heat (1).

The question of the relation of the time of ovulation to the stage of oestrus is of great importance in view of the fact that a very large number of mares fail to conceive every year. From the above evidence it will be seen that the optimum time for service should be towards the end of/
of/

of oestrus when there will be a greater chance of rupture of the follicle coinciding with impregnation. Probably the more preferable time for service would be before ovulation takes place, to enable the spermatozoa to have time to find their way upwards. However, until information is available concerning the viability of spermatozoa and ova in the horse, and the length of time taken by spermatozoa to transverse the genital tract, it will be impossible to dogmatise on the subject.

The Corpus Luteum.

After discharge of the ovum the follicle undergoes further changes. The membrana granulosa becomes thicker due to enlargement of the cells (multiplication of cells may also occur). A yellowish material is laid down within them and this part of the ovary is now known as the corpus luteum. Stroma tissue and blood vessels grow in from the theca interna, accompanied by theca cells which are large epithelium-like cells derived from the theca interna. These cells also come to contain a yellowish material and are indistinguishable from those derived from the membrana granulosa. The further/

further development of the corpus luteum depends on the occurrence of fertilisation. If fertilisation does not occur, it ceases to develop, in man, in about 19-20 days after the beginning of the cycle and in about 27-28 days undergoes regressive changes, ultimately disappearing. The remains of the corpus luteum eventually become fibrous and is known as the "corpus albicans". If fertilisation occurs and pregnancy ensues, the corpus luteum persists for a considerable time, in man until the twelfth week of pregnancy. Ultimately this also is absorbed into the ovarian stroma.

In rodents during oestrus certain follicles undergo growth, become much larger during pro-oestrus and rupture during oestrus. In the rat ovulation is stated to occur any time, 18 hours after the beginning of cornification of the vaginal epithelium. Corpora lutea now develop and attain their greatest size about 3 days after oestrus. Growth of follicles does not occur during pseudo-pregnancy or pregnancy, or at least until after atrophy of the corpora lutea has taken place a short time before the end of either period.

Seaborn and Champy state that in the mare the corpus/

corpus luteum is well formed 6 days after the acceptance of the male (67). From the 8th-14th day, no signs of further development are seen and from the 16th-19th it is undergoing regression. There is thus an alternation between the periods of maximal development of the Graafian follicles and the corpus luteum. According to Aitken the corpus luteum attains its greatest size by the 2nd-4th day and soon starts reducing slowly. Also he states, it is much reduced in size before the middle of gestation.

The Uterine Cycle.

The menstrual cycle in woman is usually divided into four stages: (1) the constructive stage, (2) the destructive stage, (3) the stage of repair, and (4) the stage of quiescence. The uterine mucosa during the resting stage consists of an intact layer which dips downwards in the form of tubular glands. This stage lasts about 12 days. During the constructive stage, which lasts about 5 days, the endometrium becomes thickened, the glands enlarge, the stroma cells proliferate and the capillaries become congested. The destructive stage/

stage or the stage of menstruation, which lasts about 4-5 days, is characterised by the passage of blood to the outside. The capillaries break down and blood accumulates in the stroma, until it finds its way into the uterine cavity through the disintegrating mucosa. It passes to the outside with the cast off mucosa cells, leucocytes and mucus from the uterine glands. During the stage of repair, which lasts about 7 days, the blood in the stroma is absorbed, the remaining cells of the surface epithelium proliferate and the uterus gradually returns to its resting stage. The average length of the menstrual cycle is 28 days.

The changes in the non-pregnant uterus in the bitch have been similarly divided into four stages. The period of rest is regarded as corresponding with anoestrus, the period of growth and destruction with pro-oestrus and the period of recuperation with oestrus. This stage in the bitch is followed by pregnancy or pseudo-pregnancy depending on the occurrence of fertilisation. The changes which occur in pseudo-pregnancy are very similar to those of pregnancy but of a less degree. Uterine changes have been described in the sheep and/

and the sow on similar lines (56). No extensive study has been made of the mare. Stockard and Papanicolaou (75) have described the oestrous cycle in the guinea-pig, and Long and Evans in the rat (52).

In rodents the uterus is constricted during dioestrus, during pro-oestrus an enlargement takes place, which reaches its maximum during oestrus. This increase in size is due to accumulation of fluid in the lumen. Regression occurs during metoestrus and the uterus gradually resumes its dioestrous state. Growth takes place during pseudo-pregnancy. It is interesting to note that haemorrhage may take place at the end of this stage (61c).

Fertilisation.

Fertilisation is made possible by the ejaculation of seminal fluid into the female genital passages. The spermatozoa find their way through the cervix into the body of the uterus, in some cases aided by the rhythmic contractions of this organ. Thence they are carried up the uterine tubes by the lashing of their tails. It is in the tubes that the meeting between the spermatozoa and the ovum or ova usually takes/

takes place and fertilisation is accomplished by the entry of a single spermatozoon into each ovum. Occasionally, however, two spermatozoa may gain entry to an ovum, resulting in the formation of twin embryos. Thereafter the fertilised egg or zygote descends into the uterus and ultimately, if pregnancy ensues, becomes attached to the uterine mucosa. The time of attachment of the ovum varies in different animals.

Implantation of the ovum.

When fertilisation occurs and the zygote becomes embedded in the hypertrophied uterine mucosa the cyclical phases of development and degeneration are disturbed. In woman the decidua is not cast off but undergoes further development in connection with the ovum.

The ovum eats its way into the decidua which closes over it. The decidua is now regarded as having three parts: (1) the "decidua basalis" between the embryo and the muscular wall of the uterus, (2) the "decidua reflexa" between the embryo and the uterine cavity, and (3) the remaining part, the "decidua vera". The foetus thus lies between the decidua basalis and the decidua reflexa. In a short time/

time foetal membranes form round it. The outermost layer, the "chorion", is covered with vascular villi which dip into the decidua basalis. Inside the chorion is the "amnion" which completely surrounds the embryo. This contains the amniotic fluid in which the foetus floats. Its function is protective. After a time the umbilical cord is formed, which attaches the foetus to the inner surface of the chorion. With the growth of the foetus the uterine cavity is eventually obliterated owing to the approximation of the decidua reflexa with the decidua vera. A large number of blood spaces are formed in the decidua basalis in the form of cotyledons. These are permeated by the chorionic villi and the placenta is formed by this junction between the chorionic villi and the decidua basalis (31).

No decidua exists in the domesticated animals except in a modified form in the dog and cat. Where there is no decidua, the chorionic villi do not project into blood sinuses in the uterus but find their way into crypts in the mucosa. In woman, the allantois, another foetal outgrowth, is of/

of little importance, but in the domesticated animals the placenta is vascularised by it.

The diffuse placenta which occurs in the horse and pig is the most primitive type known. It consists of a large vascular sheet surrounding the foetus. In the cow and sheep there is the "cotyledonary" or "multiple" placenta, consisting of cotyledons which become attached to uterine cotyledons. The placenta in the dog and cat is known as "zonary" and consists of bands of villi surrounding the ovum (71a).

In woman the attachment of the ovum to the uterine wall takes place about the 8th-10th day (55f). In sheep the attachment is complete by the 30th day (55d). It does not occur until the 12th day in the cat and the 20th day in the dog (55e). This delay in attachment of the ovum does not interfere with its development for it receives nutriment from the yolk sac and the uterine secretion.

In the mare during the first 7 weeks the embryo is fixed to the lining membrane of the uterus by means of the embryonic sac. At the end of the 3rd week of gestation about one-fourth of the embryonic sac probably adheres to the uterus, but at the end of the 6th week all the grappling/

grappling structures are at one pole. The supply of nourishment from the yolk sac comes to an end about the 7th week and the future development of the embryo depends on a more permanent apparatus, namely the chorionic villi. It is not therefore, until after about the 8th week that the embryo is safely implanted (21b).

The placenta acts as an organ of foetal nutrition and excretion. In woman the foetal villi dip into the maternal blood sinuses, oxygen and food material are taken up and waste material passes out (31). In animals the "trophoblast", a vascular layer of cells on the outer membrane of the foetus, forms an attachment with the uterine mucosa and it is in this way that nutriment is obtained (71b).

During the course of pregnancy the uterus and its contents increase in size. This is due to the increased growth of the embryo and the accumulation of amniotic and allantoic fluid. The hypertrophy of the uterus is due to multiplication of muscle fibres and to their increased size. The period of gestation ends with the expulsion of the foetus or foeti at "full term".

II. THE OVARY AS AN ENDOCRINE GLAND

The effect of castration.

Gonadectomy in the female has certain well marked effects. When performed in the young subject, the normal development is interfered with. It tends to produce a type which is intermediate to the two sexes, but sometimes the balance swings towards the male side. This may follow conditions causing atrophy of the ovaries in the human subject, where the male type of trichosis tends to develop.

The most striking results of this operation in the young subject are seen in the generative organs, which remain in an infantile or comparatively undeveloped state. Oestrus never develops and the secondary sex characters fail to appear or are much modified. When this operation is performed in the adult, the results are very similar, but differ in degree because the animal has become fully developed. The Fallopian tubes, uterus and vagina undergo regression in size and partial atrophy ensues. Here again, oestrus does not occur.

It is seen from this operation that the presence/

presence of the ovaries is essential to the normal functioning of the other reproductive organs. This is believed to be due to the hormonal activity of the ovary. The interstitial cells are regarded as the source of this secretion. The main evidence in support of this contention is derived from the exposure of the ovaries to X-rays. Careful and graduated exposure causes atrophy of the Graafian follicles, but either the interstitial tissue is unaltered or else hypertrophy takes place. In animals treated in this way the uterus hypertrophies, the mammary gland becomes enlarged and the secondary sex characters and sex instincts remain normal. Destruction of the interstitial tissue is caused by too prolonged or too intense exposure.

Castration of female birds tends to produce a neutral type with a tendency towards the male type of secondary sex characters (65). Hens, in which the ovaries have ceased to function owing to old age or disease, have been known to acquire the male type of plumage. Crew describes cases of hens in which the ovaries exhibit atrophy. Such hens may be indistinguishable from cocks of the same breed as regards secondary sex characters and sex instincts. They fight with cocks, pursue and tread/

tread hens and may even be capable of fertilising their eggs.

The development of these male characteristics is regarded as being due to a rudimentary gonad, situated on the right side of the abdomen. On removal of the ovaries, this male sex gland develops and the bird assumes the male type of plumage. The presence of the ovary exerts an inhibitory effect on the development and functioning of this gland, but with its removal or by interference with its function through disease, the right gonad is free to develop. If this gland is removed after the male plumage has developed, the male characteristics disappear. This also happens when the sex gland is left in situ, disappearance of the male plumage occurring after a certain time. Crew regards this gland as being hermaphroditic in respect to hormonal secretion, the first secretion being equivalent to that of a testis, and the second to a testis plus ovary (16).

Grafting of ovaries into castrated female animals.

If certain features characteristic of female animals disappear on castration, it might be expected that these would become evident again on the/

the re-implantation of an ovary. That this is so, is shown by the following experiments. Implantation at the time of castration prevents the appearance of the signs of castration. Implantation sometime after castration causes the female characteristics to reappear. The time taken to recover the female characteristics depends on the time which has elapsed since the ovariectomy was performed. The consequences of the removal of the ovaries from women because of disease may be averted by the implantation of an ovary or a portion of one. Atrophy of the uterus does not occur and menstruation recommences. A case is on record of the birth of a child to a woman from whom both ovaries had been removed and replaced by portions of an ovary from another woman.

The removal of a graft from a previously castrated animal causes the signs of castration to reappear.

The inference is that the ovary produces a secretion on which the female sexual characters are dependent, also that its influence is in some way necessary for the normal functioning of the generative organs. This is shown by the atrophy of/

of the uterus and the cessation of oestrus on removal of the ovaries. That another secretion is concerned in birds is shown by the fact that castration is followed not only by the appearance of the female characters but also by the appearance of some of the male characters. This latter secretion has an inhibitory effect on the development of the male characteristics.

Feminisation of Males.

Further proof of the endocrine function of the ovary is shown by Steinach's experiments on rats (65b). He was able, by the implantation of ovaries or portions of ovaries into castrated animals, to produce "feminisation". This has been confirmed by other workers. Not only do the essential male organs, penis, testes, prostate and seminal vesicles, atrophy, but they are even smaller than they would have been if the castrated animal had not received the ovarian graft. This is another indication of the inhibitory action of the ovary on the male characters. The teats and the mammary gland become enlarged. If the graft is removed, the female characters disappear. The grafted/

grafted castrated male animal becomes smaller than the normal entire male and the normal entire female; that is to say, hyperfeminisation has occurred. The feminised male is treated by males as a female.

III. /

III. PHASES OF THE SEXUAL CYCLE.

In 1928 Wiesner reported on the phasic nature of the sex cycle.(79, 80). He dealt with the problem under the headings of periodicity, i.e. the recurrence of the same conditions such as bleeding, ovulation, sexual activity and the phenomena themselves. This periodicity is inherent in the lives of animals but differs somewhat in the two sexes. In the males of many animals, with the approach of the breeding season, certain changes take place such as increased vitality, differences in behaviour, as for example, the migratory impulse in birds (though this is common to both sexes) which is closely associated with the periodic growth of the sexual organs. During this season stags are in a constant state of sexual excitement and the antlers are completely developed. At the end of this period the antlers are shed. Many more instances of the periodic display of sexual activity in the male could be given, but these will suffice for the/

the purpose of illustration. ^{such} No/obvious change occurs in the sexual organs of the male of many species as in the female during this season, and often the only indication of sexual activity on the part of the male is from its behaviour when with the female. In many species the male is capable of copulating at any time and thus periodicity in the male may be said to be largely dependent on periodicity in the female.

In the lower animals, e.g. fishes and the frog, the sexual act is confined to the extrusion of ova and spermatozoa by the female and male respectively. As however, reproduction is also attained in this way the extrusion of gametes is regarded, not only as a sexual act, but also as a reproductive act. Similarly in all male animals the sexual and the reproductive functions are performed by the ejaculation of sperm. This, however, does not hold in the female of the higher vertebrates, where the two functions are distinct. The mere act of ovulation is not a sexual act and occurs independently of it in most animals. In the bat for example, the sexual act occurs in the autumn, while ovulation does not take place until the/

the spring. The separation of the two functions is to be correlated with the fact that the extrusion of gametes is no longer necessary for the further development which can now take place within the maternal organism.

The restriction of the sexual activity to certain times of the year has a definite purpose. No doubt, in the first place it was due to the necessity of meeting the demands of the young, and because the climatic and other environmental factors are more suited for their development at one time of the year than at another. This can be referred back to the time at which mating takes place. It is well known that changes in the environment often interfere with the breeding capacity of an animal, e.g. wild animals in captivity. The doctrine that sexual activity is related to certain stimuli, environmental and seasonal, lends support to the assumption that the breeding habit is restricted to certain seasons under the influence of natural selection in order to meet the requirements of the young.

It is at that time of the year at which the sexual organs exhibit special activity, that ovulation and insemination occur and by this co-ordination/

ordination of the male and female sexual functions the likelihood of fertilisation occurring is increased. A sequence of heat periods during the sexual season affords greater opportunity for copulation. Instead of co-ordinating insemination with oestrus as in the lower animals, the same end can be achieved by allowing sexual activity to take place at any time, as in man. The possibility of fertilisation occurring here is probably as great as where sexual activity is restricted. A position intermediate to those animals in which sexual activity is restricted and to those where it is unrestricted is occupied by, for example, the rabbit, in which copulation can take place at any time, but in which ovulation is consequent on mating having taken place. This probably represents the most economical type of sexual cycle.

It is in the female that changes, typical of the sexual season, occur. Preceding oestrus, the uterus undergoes hyperaemic and proliferative modifications which attain their maximal development during oestrus and degenerate at its close. This/

This may be followed immediately or after a short interval by a further development of the genital organs, a preparation for the reception of the fertilised egg. The series of changes are completed after birth when the generative organs return to their resting state.

The cycle in the lower animals thus occurs in two phases, the sexual and the reproductive. It is possible for the reproductive phase to occur even in the absence of the sexual phase as is shown in the bitch in which changes occur, following oestrus, which simulate those of pregnancy. This pseudo-pregnancy is to be regarded in the nature of a misguided preparation for the reception of the ovum; in the bitch however, it is rather unnecessarily prolonged. In the rat the proliferative changes do not occur until they are likely to be utilised and therefore pseudo-pregnancy proper can only take place after sterile coition.

The separation of the sexual and the reproductive phases is most clearly marked in marsupials in which two sets of organs are used, one during the first phase for the reception of the spermatozoa and the other during the second, through which the foetus/

foetus is born.

Wiesner correlated these phases with the internal secretion of the ovary. This he also regards as being diphasic, the first phase being the non-pregnant one, and the second, the reproductive. During the first phase the alpha or the oestrus-producing hormone is elaborated by the ovary and the occurrence of oestrus is due to its secretion. The absence of oestrus can be due to two things: (a) the occurrence of the reproductive phase, or (b) the internal, an ahormonic state. This absence of oestrus can be referred further back, to the production of the beta hormone coinciding with the reproductive phase or to transient ovarian inactivity occurring during the internal. This beta hormone is a substance which has been extracted from the corpus luteum and from the blood of pregnant animals. It does not incite oestrous changes but produces phenomena characteristic of the reproductive phase. This and other experiments dealing with the ablation of the corpus luteum lead to the conclusion that the reproductive phase is intimately connected with the beta hormone.

The/

The periodic incidence of oestrus in the unmated animal is accounted for by the occurrence of phases of alpha secretion at regular intervals, separated by phases of endocrine inactivity. The injection of alpha induces changes typical of the first phase but not of the second. It was this discovery that led to the conclusion that another hormone was concerned with the changes of the second phase and beta was incriminated. Beta, however, does not appear to be the only hormone responsible for the pregnancy changes; alpha is also found in the blood and urine during this period. Thus alpha would appear to be necessary for the second phase, although it is not able to produce it (unless beta is present) and even interrupts it when given in large doses. Wiesner regards its effects as being inverted during this phase; the oestrous changes are not produced and it functions as a hyperaemising and growth factor.

It will now be shown how the ovary is influenced by the secretions of the anterior pituitary, alpha or beta hormones being produced according to whichever pituitary hormone is acting upon it.

IV. THE RELATIONSHIP BETWEEN THE ANTERIOR PITUITARY
AND THE OVARY.

That a relationship between the anterior pituitary and the ovary exists has been demonstrated by the marked aberration of sexual function which follows disorders of the anterior pituitary. Hypopituitarism is associated with delayed sexual development and a permanent condition of infantilism may be produced. Interference with the sexual functions also occurs in hyperpituitarism. As the disease advances amenorrhoea in the female and impotence in the male are distinctive features.

Evans and Long were the first to investigate this relationship experimentally. They found that the daily injections of large amounts of anterior pituitary hormone into the normal rat resulted in the disappearance of the oestrous cycle. This was associated with the formation of atretic corpora lutea in the ovaries. After the injections ceased, the oestrous cycle appeared normally once more (20).

Zondek/

Zondek and Aschheim in 1927, from the results of extensive experiments, discovered that the implantation of the anterior pituitary into the immature mouse brought about precocious oestrus, and that this effect is produced through the ovary which undergoes maturation and elaborates the oestrus-producing hormone (82). Smith and Engle arrived at very similar results (61f). Smith found that hypophysectomy stopped the oestrous cycle (73). It would seem that the coincident elaboration of this hormone with the maturation of the Graaffian follicles was very significant. Sterilisation by exposure to X-rays and the consequent destruction of the follicles, however, does not inhibit the occurrence of oestrus in the immature animal which has been implanted with anterior pituitary.

The action of the anterior lobe of the pituitary on the immature mouse is recognised by Zondek and Aschheim as follows:-

1. Maturation of the follicles, ovulation and appearance of oestrus.
2. Formation of haemorrhagic spots on the enlarged follicle.
3. Luteinisation of the follicles and formation of corpora lutea atretica.

Whether/

Whether one or more hormones is concerned in the above changes is still a matter of some doubt. Zondek and Aschheim found that the ovulation stimulating principle is destroyed by alkali, and Evans and Long failed to produce oestrus in the immature rat by the injection of alkaline extracts of the anterior pituitary. Zondek found that he could produce the three reactions by the use of aqueous extracts given in varying proportions and that these were due to one hormone, the luteinisation obtained by Long and Evans being caused by the larger amounts present in the alkaline extracts (61f).

The more recent view, however, is that two hormones are concerned in the regulation of ovarian function. Wiesner and Crew (81) postulated the existence of two hypothetical substances, "rho" one and "rho" two. Rho one is responsible for the maturation of the follicles and ovulation, and rho two for the luteinisation and formation of blood spots. This corresponds to the later view of Zondek who also recognises two factors HVH-A and HVH-B, corresponding to rho one and rho two respectively (83).

The/

The rho two factor is easily recognised macroscopically by its effect on the ovary, but rho one can only be demonstrated indirectly by the changes it induces in the vagina and uterus through the medium of the ovary. This necessitates the removal of the oestrus-producing hormone from the substance injected.

Aschheim and Zondek have used the presence of the anterior pituitary hormone, HVH-B, in the urine for the diagnosis of pregnancy in women (6). The technique of their test is as follows. Five immature mice are used and receive 0.2 c.c., 0.25 c.c., 0.3 c.c., and 0.5 c.c. urine morning and evening for 3 days. The mice are killed on the 5th day and a positive result is recognised by the occurrence of haemorrhagic spots and corpora lutea in the ovaries. The presence of this hormone can be demonstrated in the urine as early as about the 10th day after conception which corresponds to the time of implantation of the ovum. From the 1st to the 8th week of pregnancy the amount of HVH-B found in the urine is from 3000-5000 units; from the 3rd to the 7th month 3000-6000; and from the 7th to the 9th 2000-3000.

V. HORMONES OF THE ANTERIOR PITUITARY IN THE
PREGNANT MARE.

Cole and Hart using the serum of pregnant mares were able to produce oestral conditions in immature rodents (11, 12). The reactions were uniformly negative up to the 37th day of pregnancy and the time of the first reaction varied from the 37th to the 42nd day. The first reaction was observed macroscopically in the uterus and vagina and the weight of the ovaries was found to be comparable to that of controls. After the 42nd day the serum had a marked effect on the size of the ovaries. This maximal effect was produced on the ovaries from the 42nd to the 80th day after which it gradually declined until the 180th day when the ovaries corresponded in size to those of controls. Luteinisation and the formation of blood spots in the ovary also followed the injection of serum from pregnant mares. The reaction obtained from the 37th to the 42nd day was evidenced solely on the uterus and vagina. It is/

is possible that this was brought about by the action of the maturation factor, the rho one factor of Wiesner, which stimulated the ovary of the test animal to produce the alpha hormone and hence the reaction on the uterus and vagina. In the absence of further information, however, it seems just as likely that this reaction was due to the alpha hormone of the ovary. In the period between the 80th and the 180th day the anterior pituitary hormone disappeared. Cole and Hart consider the presence of this hormone in the blood of pregnant mares may be used as a means of diagnosing pregnancy of 6-7 weeks duration. This reaction continues until the 100th day. Serum obtained from the 222nd day of pregnancy until the end of this period had an inhibiting effect on the ovaries of the test animals in so far as the weights of the ovaries of injected animals were less than those of the controls from the same litter.

The correlation between the work of Cole and Hart and Zondek is of great interest. Zondek (85) found that the amount of anterior pituitary hormone on the blood of the pregnant mare up to the 100th day is about the same as that in the pregnant woman, namely 4,000 mouse units per litre. Sera from other/

other mares later in pregnancy gave slighter reactions and when taken from mares in the latest stages of pregnancy, none at all. It would thus seem to be fairly well established that the anterior pituitary hormone, HVH-B, occurs only in the earlier stages of pregnancy in the mare, probably up to about the 100th day.

Zondek stresses the fact that HVH-B is not eliminated at all in the urine of the mare, while HVH-A is excreted only in small quantities during the first months of pregnancy. The average amount of this hormone per litre of mares urine is given as 800 rat units.

The diagnosis of pregnancy by the demonstration of folliculin and the ovulation hormone was investigated by Zondek. The period of pregnancy covered was from 74-260 days after mating. The test was carried out on infantile rats as the urine was too toxic for mice. Normal immature rats were used to allow of the expression of the ovulation hormone. Diagnosis was made by means of vaginal smears. In an examination of 80 mares a 2.5 per cent. error was obtained (85).

It would seem to be necessary that any investigation/

investigation of the early stages of pregnancy in the mare should take account of (1) the excretion of the oestrus-producing hormone in such small quantities that it may not be possible to differentiate between this stage and the oestrous cycle, (2) the fact that it may not be possible to make use of this hormone for some considerable time after conception, and (3) that the ovulation hormone may be determinable at an earlier stage than the oestrus-producing hormone.

With this end in view the urine of pregnant mares was examined for the ovulation hormone and concurrently for the luteinisation hormone.

From the time of mating onwards regular and frequent specimens of urine from a Clydesdale mare and several Shetland ponies were examined for the presence of anterior pituitary hormones. It was not possible to apply chemical methods to the separation of the alpha hormone from the urine, so this investigation was necessarily limited to the question of determining if the alpha factor appeared in the urine of the pregnant mare before the alpha hormone and also, to discover if rho two, the factor on which the human pregnancy test is based/

based, was present in the urine.

All specimens of urine taken from the above animals were injected into immature and ovariectomised animals in similar amounts to those used in the human pregnancy diagnosis test. Vaginal smears were made on the 5th day of the test from the castrated mice and the immature mice were killed and their ovaries examined for blood spots and corpora lutea. Specimens of urine from the 16th-140th day of pregnancy were examined and also specimens taken at intervals throughout gestation.

In no case was the rho one factor of the anterior pituitary determinable before the alpha hormone, cornification always developed concurrently in both lots of mice. No blood spots nor corpora lutea were observed in the ovaries of test animals at any period of pregnancy.

VI. /

VI. THE DISTRIBUTION OF THE OESTRUS-PRODUCING
HORMONE.

The earlier workers on this subject used the prevention of uterine atrophy which follows double ovariectomy, as a test for the activity of their ovarian extracts. These extracts were very variable in their action and the saline extracts used at this time were not very effective. Marshall and Jolly (1906), by the injection of saline ovarian extracts into a bitch, were able to produce oestrous changes (56). In the following year, Sonnenberg (74) was unable to produce oestrus by the injection of liquor folliculi.

It was not until 1912 that any definite advance was made. The difficulties confronting early workers were twofold: (a) a lack of knowledge concerning a satisfactory method of testing the activity of their preparations, and (b) the difficulties concerned with the preparation of such extracts. At this time the method in vogue for testing the activity of extracts depended on the/

the production of hypertrophy of the uterus in the test animal. In addition, growth of the mammary gland was caused by active extracts. Iscovesco in 1912 produced a lipoid extract of the whole ovary of the sow which caused marked uterine hypertrophy and growth of the mammae in the rabbit (36). A similar result was obtained from lipoid extracts of the placenta (36). Fellner in 1913, using ovariectomised animals, showed that oestrous changes were produced by extracts obtained from the placenta, the ovaries after removal of corpora lutea and follicles (29). Herrman and Fraenkel used immature animals as test animals and demonstrated the presence of the oestrus-producing hormone in the placenta (52). Frank and co-workers describe the occurrence of this substance in the placenta and corpora lutea (22). That this hormone is universal in its distribution among animals was shown by its discovery in the ovary of the cow by Herrman, in the bitch by Frank, and by Allen and Doisy (4) in the human ovary. Herrman also observed it in the corpora lutea of the sow, Frank and Rosenbloom in the cow (26), and Allen and Doisy in human corpora lutea (22c).

In/

In 1922 Frank published the results of his experiments with follicular fluid obtained from the ovaries of cows, pregnant and non-pregnant (22b). The fluid was injected into rabbits and well marked hypertrophy of the uterus was noted. In the following year Seaborn and Champy carried out similar experiments with the follicular fluid of the mare (67). They injected the fluid into rabbits and were able to demonstrate the active substance in the follicular fluid taken from a mare during oestrus during the stage of heat but not in the follicular fluid of a mare which was not in heat. It is now known, however, that follicular fluid is active irrespective of the stage of the oestrous cycle. Frank obtained positive results from the injection of follicular fluid from non-pregnant cows.

A great advance was made in 1923, in the technique of testing the activity of the oestrus-producing substance, by Allen and Doisy who originated the vaginal smear method for the recognition of the cyclical changes in the vagina of rodents (4). This method was made possible by/

by different workers who investigated the cyclical changes in various rodents, Morau in the mouse (59), Retterer and also Konigstein in the rabbit and guinea-pig (64), Stockard and Pananicolaou in the guinea-pig (75) and Long and Evans in the rat (52).

It will be of advantage to describe the changes in the vagina of rodents at this point. During pro-oestrus the vaginal mucosa undergoes growth. Later cornification of these cells occurs and they are cast off. This coincides with the stage of oestrus. Allen and Doisy made use of the changes which occur in the vaginal epithelium for the demonstration of the activity of the oestrus-producing hormone. Their method consists of making a smear of the vaginal contents and examining it microscopically.

During dioestrus the smear consists of nucleated epithelial cells and leucocytes. The leucocytes gradually disappear from the smear, until at pro-oestrus it consists of nucleated epithelial cells. As this stage advances cornified cells begin to appear and when oestrus occurs they are the only type of cell present. This stage ends with the infiltration of leucocytes (metoestrus) and/

and the vaginal cells gradually resume their normal resting condition.

The discovery of a simple and practical method of determining the various stages of the oestrous cycle in the mouse and rat was a milestone in the investigations on the oestrus-producing hormone. The work had received a new impetus and a large number of workers were attracted to the field. Allen and Doisy, by the injection of liquor folliculi from the cow and pig, were able to produce oestrous changes in castrated rats and mice. They concluded that this fluid was the source of the hormone. They failed to find it in the corpora lutea of the lower animals, but later they demonstrated it in human corpora lutea, in the early stages of their development (4). Courrier (13) in 1924 produced oestrous changes in the guinea-pig and rabbit by the injection of follicular fluid and follicle extract, and in the following year Frank and Gustavson obtained the oestrus-producing substance from the corpora lutea of hogs and cattle (23). They maintained that the follicle secreted the female sex hormone up to the/

the time of ovulation after which the corpora lutea continued the secretion until it was assumed by the placenta. They called this triad - follicle, corpus luteum and placenta - the "Gectational Gland". Conflicting results concerning the presence of this hormone in the corpus luteum have been obtained. Positive results were obtained by Pratt and Allen (22k) in addition to workers already mentioned. Others, however, failed to find it. This discrepancy is explained by the fact that many corpora lutea contain a fluid derived from the liquor folliculi, which contains oestrin. This finds support in the discovery of Parkes and Bellerby, that the fluid in the corpora lutea of the cow contains this substance, while the solid corpora do not (63). It may therefore be said that the oestrus-producing hormone occurs in the ovaries of all animals examined; (1) in the follicular fluid, and (2) in the ovarian stroma, and that its occurrence in the corpus luteum is probably incidental.

The presence of this hormone in the placenta has been shown by many workers (61e). Parkes and Bellerby/

Bellerby (62), Allan, Dickens, Dodds and Howitt (2), have demonstrated it in the human placenta, Parkes and Bellerby in the cow, and Aschheim in the sheep (22). The foetal membranes, amniotic fluid and umbilical fluid have been found to contain this hormone. Parkes and Bellerby, discussing the significance of the occurrence of oestrin in the placenta, state that its distribution in the placenta and foetal fluids supports the view that it is merely absorbed from the maternal circulation by the placenta rather than produced by it (62).

The oestrus-producing hormone in the blood.

Marshall and Jolly in 1905 claimed to have obtained immediate ^{swelling} of the vulva in the anoestrous bitch by the injection of blood from a bitch which was in heat (56, 57). Schickele (66) in 1913 failed to produce uterine growth in rabbits by injecting blood from pregnant rabbits. Fellner in similar experiments obtained doubtful results (30).

As the blood would be the normal method of transmission of the hormone from its site of origin/

origin to the site of activity, its presence there ought to be capable of demonstration. This proved to be the case. Also, as the sexual cycle is periodic in its appearance, it would be expected that so also would be the oestrus-producing hormone. Loewe (47) demonstrated this hormone in the blood of women during the menstrual cycle and Frank, Frank, Gustavson and Weyerts (24) in the same year showed that it occurred in the blood of oestrous sows and was absent in the anoestrous animal and in the bull. Smith dealt with its occurrence in the blood of the non-pregnant woman (72). Loewe states that during the premenstrual period the female organism is flooded with the ovarian hormone. The concentration drops just before menstruation and is almost absent during the menstrual period. It occurs in the menstrual blood in a greater concentration than in the circulating blood. Frank found that the oestrus-producing hormone was present in greatest amount in the blood during the five days previous to menstruation. An abrupt rise then takes place but immediately after the onset of menstruation an abrupt drop regularly occurs. The first menstrual/

menstrual blood contains a large amount of female sex hormone in concentrated form, but thereafter a rapid drop occurs.(22d).

Binz in 1924, by the injection of blood serum of pregnant women into immature mice, produced signs of premature puberty (8). It was found to exist in large amounts in the blood during pregnancy by Fels (28) and Trivino (77) in 1926, and by Zondek and Aschheim in 1927 (83). Trivino injected serum from pregnant women into immature mice and obtained enlargement of the uterus. Fels found the concentration to be markedly increased after the 6th month. Aschheim noted this increase after the 4th month. Frank and co-workers describe a high hormone level in the blood from the 7th week of pregnancy to the term. Hirsch (1928), using Frank's technique, discovered a substance in the blood that led to typical oestrous changes in the vagina of mice (33). After the termination of pregnancy the hormone dropped rapidly within 24 hours. That the rapidity at which this hormone disappears from the blood is intimately connected with the separation of the placenta, is instanced by a case of Frank's in which the placenta/

placenta remained in utero for 17 days after delivery of the child. This was associated with a high concentration of the hormone in the blood, and this concentration was maintained until the placenta was removed.

Cole and Hart investigated the blood serum of mares for sex hormones (11, 12). They obtained negative results with blood taken from different mares, 3, 5, 12 and 16 days before oestrus. After mating, reactions were uniformly negative up to the 37th day of pregnancy. The first reaction was observed from the 37th-42nd day in the uterus and vagina. (The effect of the serum on the ovaries has already been noted). Rats were used as test animals. At this time, however, the weight of the ovaries of the test animals was comparable to that of controls. It would seem therefore that this reaction was due to the oestrus-producing hormone although the possibility of there being an amount of anterior pituitary hormone in the serum sufficient to stimulate the production of ovarian hormone in the test animal must be considered. Vaginal reactions were obtained until the end of pregnancy. A fraction of a c.c. of serum was found to be capable of producing a positive vaginal/

vaginal reaction in the early stages, whereas in the later stages about 25 c.c. were necessary. This implies either that the concentration of the hormone has diminished or that there is a substance which inhibits its expression. Serum from a mare, 121 days pregnant, produced a positive vaginal reaction in ovariectomised rats, but apart from this instance immature rats seem to have been used entirely. They obtained ovarian reactions from the 40th-80th day of pregnancy approximately and after this there was a diminution to the 180th day. Thereafter the effect appeared solely on the uterus and vagina. It is highly desirable that ovariectomised animals should be used for the demonstration of the oestrus-producing hormone, particularly if no chemical method of separation of this hormone from the anterior pituitary factor is employed. Whether the oestrus-producing hormone is present throughout the period from about the 37th to the 180th day (when the anterior pituitary factor can no longer be demonstrated) cannot be decided at present. The sole evidence is the injection of serum from a mare on the 121st day of pregnancy into castrated rats. The inference, however, to be drawn from their work/

work is that the oestrus-producing hormone is present in the blood of the mare from about the 37th day of pregnancy until parturition, after which it disappears, although direct experimental evidence would still seem to be lacking.

The oestrus-producing hormone in the urine.

Following the discovery of this hormone in the blood, attention was directed to the possibility of its excretion in the urine. Loewe and Lange examined the urine of women during the menstrual cycle and found that the ovarian content is almost nil a few days preceding, during, and a few days after menstruation (48). The concentration rises rapidly between the 3rd, and 10th-11th day after menstruation and reaches its maximum concentration about the 10th-11th day. Laquer reports as much as 200 mouse units per litre on the 2nd day of menstruation, but there is a possibility that contamination with menstrual blood may have occurred (22 i).

Zondek, in 1927, found large amounts of the oestrus-producing hormone in the urine of women during pregnancy (82). Aschheim and Zondek state that/

that it is doubtfully present during the first two months and from about the 8th week onwards large amounts are found (6). It is interesting to note that the appearance in the urine coincides with its appearance in the blood. Frank (22f) states that the blood does not contain this hormone until approximately the 8th week after impregnation. Aschheim and Zondek found 300-600 mouse units of oestrin during the 1st-8th week of pregnancy, 5000-7000 from the 3rd-7th month, and 6000-10,000 from the 7th month to the end of gestation (6). Veler and Doisy (1928) confirmed this (78). The rapid disappearance of the hormone from the urine after parturition was noted by Aschheim and Zondek.

The following table is taken from Veler and Doisy.

<u>Time, post partum (hours)</u>	<u>Rat units per litre</u>
8.5	640
37	500
48	80
72	22
96	20
144	5
192	5
264	4

Mazer/



Mazer and Hoffman in 1929 attempted an early diagnosis of human pregnancy by the injection of 20 minims of urine every 2 hours for five times into castrated mice (58). This test gave an error of 8 per cent. Zondek and Aschheim do not regard the ovarian hormone as being suitable for the demonstration of pregnancy because its presence in the urine even in considerable quantity is not specific for that condition; it may be found in the urine during the menstrual cycle and in disturbances of ovarian function. Frank found the oestrus-producing hormone in the urine of pregnant women but in lesser amounts than did Aschheim and Zondek (22f).

Aschheim and Zondek in 1927 demonstrated the oestrus-producing hormone in the urine of cows (83), as did Hisaw and Meyer who found 650 rat units per litre (35), Nibler 12-148 rat units per litre (60), and Frank 2-400 units (27). Lipschutz and Veshnajokov state that the amount of oestrin derived from the urine of the pregnant cow is very small - at the most it amounts to 800 mouse units per litre. No increase was determined during the course of pregnancy (45). Turner, Frank/

Frank, Lomas and Nibler (1930) found that the secretion of this hormone was at a low level in the early stages of gestation, but that the secretion gradually increased until the time of parturition (76). This latter finding is what one would expect.

Zondek has investigated the urine of mares 74-260 days after mating for this hormone. He found exceedingly large amounts, namely, an average of 100,000 mouse units per litre (85). Kust (40) on the other hand gives the hormone content of the urine as from 2-7 units per 1 c.c. from the 3rd month onwards. The secretion of ovarian hormone was found to increase as pregnancy progressed. In the first 5 weeks the secretion does not always amount to 1 unit per 1 c.c. From the 6th-10th week the amount is always more than 1 unit and up to 2 units, and from the 3rd month onwards as stated above. During oestrus up to 1 unit may be found in 1 c.c. of urine. No demonstrable hormone was discovered in 1 c.c. of urine of non-pregnant mares, nor in virgin mares in the inter-oestral periods. He states that a mare may be assumed to be non-pregnant if two months after the last service, less than 0.8 units are present and after a/

a further test two months later, i.e. four months in all, less than 1 unit is found. The maximum amount found by Kust was 7,000 units as compared with 100,000 units found by Zondek. A mare is diagnosed as pregnant by Kust when 6 weeks after mating the hormone content per 1 c.c. of urine is more than one and after 10 weeks more than two. It should be noted that this period of 6 weeks corresponds with the time at which Cole and Hart (11,12) obtained an increase in the size of the uterus and vagina of test animals, following the injection of serum from pregnant mares.

Amounts of less than 1 unit were found in that period of pregnancy before the 40th day. The possibility of determining pregnancy from the thickness of the uterus of the test animal was examined. At the height of oestrus the uterus (mice) was always 2-2.5 mm. thick, while towards the end it measured 1.1-1.8, and never less than 1 mm. In the infantile mouse it was found to be 0.4-0.5 mm. Zondek, however, states that the growth of the uterus which occurs during the reaction cannot be used diagnostically because it occurs too irregularly. Also, greater amounts of hormone/

hormone are necessary to produce an oestral reaction in the uterus than in the vagina.

The general indication is that pregnancy in the mare cannot be diagnosed by the presence of this oestrus-producing hormone in the urine before approximately 40 days after service.

Kust found that the hormone was absent from the urine 4 days after foaling or abortion.

Occurrence in the male.

The occurrence of the oestrus-producing hormone in the male has been reported by many workers. Laquer and de Jongh noted it in the testis (43). Allen and co-workers, however, did not confirm this (5). Hirsch states that it can be isolated from 35 c.c. of human male blood (33). Frank and Goldberger also obtained positive results from male blood (25). Loewe, Voss, Lange and Wahner, found both testicular and female sex hormone in human^{male} urine and claim to have separated them (51). R.T. Frank (22 k) discussing the significance of the isolation of this hormone from male urine, regards the high hormone content of the bile as a protective mechanism by which the/

the alpha hormone is absorbed through the portal system and stored in the bile, until the concentration becomes too great, when it is excreted through the intestine and urine. It may appear, transiently, in the blood. The question is raised, whether this hormone may have been taken in with the food. The alpha hormone is resistant to the action of enzymes and has been isolated from many vegetable substances. Lipschutz and Veshnajokov (45) remark on this possibility when dealing with the presence of alpha in the urine of cows. While it seems extremely probable that some at least of the alpha hormone, in the urine may have been introduced per os, the evidence, so far, is incomplete.

Occurrence in Faeces.

As elimination of the oestrus-producing hormone occurs in the urine it might be expected that the alimentary tract would form another means of exit from the body. Dohrn and Faure (19) are the only workers who have examined this possibility. They found very large amounts, 30,000 mouse units per kilo of dry faeces.

Occurrence/

Occurrence in Bile.

The oestrus-producing hormone has been described in male and female bile by Gsell-Busse. Frank believes that the high female sex content of bile is due to a protective mechanism by means of which the hormone is absorbed through the liver and stored in the bile until the concentration becomes too great, when it is excreted through the urine and intestine and may, transiently, appear in the blood (22 h).

Occurrence in Plants.

An oestrous reaction has been obtained from extracts made from vegetable sources. It has been described as occurring in the buds of willows (Loewe, 47), sugar beet, rice and wheat. Dohrn, Faure and Blotevogel found it in yeast and potatoes (18). A very guarded view of these findings is expressed by Parkes, who emphasises the importance of careful confirmation of such results, especially as different workers accept different criteria as evidence of a positive result.

Chemistry/

Chemistry.

The chemistry of the alpha hormone is reviewed at some length by Frank (22 g). The earliest work was done with aqueous extracts and press juices and comparatively little information was gained. The most fruitful period was that in which lipoid soluble preparations were used, and of late, water soluble preparations have come to the fore. It is not possible to enter into the details of the preparations of these extracts here, and we will therefore confine ourselves to a brief outline of the results obtained. The aim of investigators was to obtain the active principle in a form as concentrated and as free from impurities as possible, and also to determine the chemical nature of the substance involved. Taken as a whole the work done has been rather inconclusive. It is now possible to prepare a substance which contains the active principle in a relatively pure state, but very little advance has been made towards the realisation of its chemical nature. Veler and Doisy obtained from 480-1240 rat units per litre of urine from pregnant women (78). The most active preparation gave 0.06 mg. solids per rat unit. Lipschutz/

Lipschutz isolated an active fraction containing 1 mouse unit to 0.15 mg. dry substance. The amount of solids per mouse unit has been reduced to 0.1 to 0.001 mg. in the water soluble preparations compared to 0.5 mg. in the lipoid fractions.

The results of experiments emphasise the great stability of the substance. Pregnant urine retains the active fraction almost indefinitely. It is resistant to boiling in urine either in strong acid or alkaline solutions. It is soluble in water and lipoid solvents. Simonnet in 1927 isolated a substance which contained C, H and O, but no P nor N (70). It was sensitive to oxidising agents but resistant to heat and was not related to cholesterol. Dickens, Dodds and Brinkwater obtained a white powder from pigs' ovaries which was soluble in water and could be boiled in aqueous solutions (17). The active principle passed through a collodion membrane and the substance contained sulphur, histidine and tryptophane.

The work accomplished up to date merely serves to indicate that it is possible to obtain the/

the active principle in fairly high concentration and the resulting composition of the substance reflects the degree of purity obtained. Frank states that all aqueous solutions lose their activity in the course of a few days. Marrian after extraction and purification of pregnancy urine obtained an average of 51 mouse units per c.c. (54).

Actino-activity of Oestrin

Jordan and Doisy found that diffuse daylight had a destructive effect on the hormone. The destruction was more rapid in ultra-violet light (38). Ludwig and Rees (53) confirmed this and found in addition that exposure to red rays led to a twofold increase in the activity of oestrin as tested in the mouse and that such exposure restored activity which had been destroyed by ultra-violet radiation. X-radiation is without effect on the hormone.

Standardisation of the Unit.

Most animals exhibit a difference in their physiological reactions to various pharmaceutical substances. The alpha hormone is no exception to this. A great individual variation in response has/

has been found. One of the factors which complicates the estimation of the hormone is the adoption by different investigators of different criteria as evidence of a positive reaction. Loewe uses a percentage count of leucocytes (49). Frank and Goldberger (25) have a scale representing all types of reactions found.

- 0--- leucocytes, nucleated epithelial cells. Negative
- 1--- predominance of leucocytes, increase
in nucleated epithelial cells..... Negative
- 2--- occasional leucocyte, nucleated
epithelial cells..... Weak positive.
- 3--- no leucocytes, nucleated epithelial
cells..... Threshold reaction
- 4--- epithelial non-nucleated scales
exclusively..... Strong reaction.

From the wide range of positive reactions in this scale it is obvious that it can only be rough and ready, as the authors themselves recognise. Laquer (41) bases his estimation on the types of cells present in the vagina: leucocytes must be reduced to a very few with at least equal presence of nucleated and non-nucleated cells. The same objection applies to this method.

The only definite standard is that of Allen and/

and Doisy who require full cornification of the vaginal cells for a positive result (4). As this method is not subject to a cell count nor to varying proportions of different types of cells, variation in the recognition of the stage is eliminated and the only variation that can occur comes from the differential response of different animals. Allen and Doisy define the rat unit as the minimum amount of potent substance necessary to produce a full change from the negative to the oestral vaginal spread in a castrate rat of approximately 140 gm. (\pm 20 gm.) in weight.

It may be that the intervals at which the injections are given affect the response. Allen and Doisy (4) and Frank and co-workers(25) give three injections one day; Coward and Burn (14) one dose and Biedl three doses at 12 hour intervals. Frank states that a large dose brings about a positive reaction more quickly than a smaller one. Allan, Dickens, Dodds and Howitt (2) and Marrian and Parkes (61 e) found that a given amount will produce much less effect in one than in a series of injections. This is probably due to the repeated stimuli applied to the genital tract, whereas when only/

only one injection is given the hormone is eliminated from the body and the stimulus ceases comparatively soon. With the earlier fat-soluble preparations no difference was found irrespective of whether the dose was given in one dose or in several, but with the purer fat extracts and water soluble preparations, less effect was obtained from a single injection. This is considered to be due to its rapid absorption and destruction in the blood when given in a comparatively pure form. Similarly, the more prolonged effect of a single injection of the cruder fat extracts is probably due to delay in absorption.

Bugbee and Simond studied the effect of weight on the amount of this hormone required to produce a positive reaction (10). They showed that, as an animal gets older and heavier, more hormone is needed to produce a positive reaction. They used the following formula to correct variation due to weight.

$$\text{R.U. per c.c.} = \frac{W}{140} Q$$

W = weight of rat in grams.

Q = minimal no. of c.c. that will produce oestrus.

It is still problematical whether the time
at/

at which testing takes place after the operation has any effect on the reaction. Parkes states that it seems probable that the degeneration of the organs that follows the operation would result in an "entire lack of sensitivity to ovarian reaction and it is necessary to consider how far this degeneration can go without invalidating any tests done on such animals". Allen and collaborators found that the duration of anoestrus following the operation had little effect on the dose required to produce oestrus. On the other hand, Kahnt and Doisy prime their animals before carrying out a test (39). The latter workers have drawn up a method for assaying the hormone, the nature of which is so complete that it renders its accomplishment in routine procedure, well-nigh impossible.

It will readily be understood that, as individual animals show a variation in response to the injection of a given amount of the substance, the larger the number of animals used, the more accurate will be the estimation. Coward and Burn regard the unit as the amount necessary to produce oestrus in 50 per cent. of a group of ovariectomised animals (14). Laquer requires 75 per cent.

Coward/

Coward and Burn use a group of 20 animals, but with this number Dodds and co-workers found that different groups gave a different percentage reaction to the injection of a given amount. Also, a group may show a different response at different times. It seems, that, as yet, it is not possible to devise an accurate method for the assay of this hormone.

Nomenclature.

A great variety of terms are applied to the oestrus-producing hormone (22 i). This has arisen because of the tendency of investigators to name their active substance after the tissue from which it was derived or according to its supposed function. The result is a vast confusion of terms, all applied to the same active principle.

The earliest terms were those indicative of derivation, such as "ovarin" (Poehl), "oophorin" (Landau), "biovar, protovar, luteovar"(Okinschita). "Sistamensin" was applied to the product of Sietz, Wintz and Fingerfur who believed that it caused the menses to subside, and "agomensin" to the fraction which stimulated the menstrual flow. Schickele, Aschner, Frank and others called the active/

active fraction "corpus luteum" or "placenta"-containing hormone. Klein used the term "folliculin" which is still used by some workers. Glimm and Wadehn term the active fraction "feminim". "Gynacin" has also been applied. Allen and Doisy refer to the "ovarian", "follicular " or "oestrous" hormone and Loewe also uses this latter term. Frank and Gustavson speak of the "female sex hormone". The use of this term, however, implies that it is responsible for all the female sex characteristics, which has yet to be proved. Laquer calls the active fraction "menformon" and Zondek and Aschheim, the "ovarian hormone", implying that only one exists. Parkes and Bellerby refer to "oestrin" and Steinach to the "cyclical" hormone. Loewe uses "lykinin", and "tokokinin" is suggested by Blotevogel, Dohrn and Poll. Wiesner suggests the use of the term "alpha hormone" or factor, in view of the comparatively little knowledge that exists as to its nature; it does not fulfil all the functions that are attributed to the ovary; for one thing the question of its relation to mating and metabolism is still obscure.

VII. /

VII. INVESTIGATIONS ON THE OESTRUS-PRODUCING
HORMONE IN EQUINE URINE.

This work was commenced in October 1930, when all the available mares had been pregnant for at least three months. It was therefore not possible to investigate the early stages of pregnancy at this time and this question was consequently delayed until the beginning of the next breeding season.

A supply of urine was obtained in the first place from a herd of Shetland ponies, most of which had been mated in the previous sexual season. As these mares were comparatively few in number, it became necessary to consider ways of collecting a large number of specimens from other sources, in order that an extensive trial of this method might be made. Accordingly it was decided to get into touch with veterinary surgeons by personal letter and by a letter which appeared in two veterinary publications. The majority of samples were obtained in this way. Other specimens were sent in/

in by people who kept brood mares, to find out if their mares were pregnant.

In order that a constant and regular supply of urine might be available, a young pregnant Clydesdale mare was purchased by the Institute of Animal Genetics. By the collection of urine from this mare and from the Shetland ponies, it was possible to examine the excretion of the alpha factor throughout the whole period of gestation.

Judging from the enquiries received, the knowledge that such a test was being experimented with, has aroused considerable interest. Information regarding the test has been sought from as far afield as India and America. The fact that samples of urine from such places would be some time in transit would not affect the result of the test, as the hormone is capable of remaining active for some time. Specimens have been received from India; they were over four weeks old before the test was carried out, but this in no way affected the results.

The British Blood Stock Agency became interested in the problem in view of its possible value to breeders of horses and undertook to supply us/

us with a large number of specimens of urine from different mares. As these specimens were, for the most part, from the early stages of pregnancy they were especially valuable.

Specimens of urine were usually sent in without the addition of any preservative except those which were going to be some time in transit, when it was recommended that a little toluol be added. This has no effect on the reaction. Those who sent in specimens were asked to fill in on sheets supplied, particulars as to the date of service, date and time of collection of urine, etc.

(a) Description of the test.

If urine from a mare is injected into mice without any previous treatment it very often proves toxic, and some, if not all of the mice die. This usually happens before the end of the test and no result is obtained. If, however, sulpho-salicylic acid is added to the urine preparatory to injection, as is done with human urine in the Pregnancy Diagnosis Station here, this is prevented. The sulpho-salicylic acid precipitates any protein, should it be present, and detoxicates the urine by reason of its antiseptic action. Hormones in urine/

urine are not precipitated by it in the absence of protein and only small fractions are removed in the presence of protein. The mortality in the test mice when the urine has been treated in this way, is exceedingly small, being about 1 per cent. and most of the deaths occur in mice under two months old.

The amount of sulpho-salicylic acid added is 1 gm. to 25 c.c. of urine. The urine is then filtered and neutralised with sodium bicarbonate, using B.D.H. Universal Indicator, after which it is ready for injection.

Ovariectomised mice are used in this test. Immature female mice may be used also, but as the action of the alpha hormone is limited to the uterus and vagina, it is more satisfactory to use mice from which the ovaries have been removed, thus obviating any possible error arising from the ovaries of the test animal. Moreover, the expenses of the test are reduced as it is possible to use the same mice repeatedly.

The mice were usually ovariectomised at the age of from 4-6 weeks and allowed at least a week in which to recover from the operation before they were/

were used. After each test a week is allowed to elapse before they are used for a further test. A record was kept of the date on which they were ovariectomised and the number of times they were used.

For the ordinary test 4 mice were used. They were injected night and morning for 3 days with urine diluted 1-3 with normal saline solution, i.e. they receive altogether 6 injections of urine. No. 1 receives 0.2 c.c. of urine at each injection, or 1.2 c.c. in all; No. 2 receives 0.25 c.c. or a total of 1.5 c.c.; No. 3 receives 0.3 c.c. or a total of 1.8 c.c.; and No. 4 receives 0.5 c.c. or a total of 3.0 c.c. On the 5th day following the beginning of the test, smears are made from the vagina, stained with Giemsa and examined microscopically.

The vaginal smear exhibits cyclical changes in its cell content, corresponding to the different stages of the oestrous cycle. The changes in the character of the vaginal smear, consequent on the injection of urine from pregnant and non-pregnant mares is shown in the accompanying photomicrographs. Only complete cornification of the vaginal epithelium is regarded as evidence of a positive result.

The/

The injection of the above amounts of urine covers approximately from 1000-2500 mouse units of this hormone per litre. (A mouse unit is defined here as the smallest amount of the active substance in equine urine, which, given in 6 doses, distributed over 3 days, will produce complete cornification of the vaginal epithelium in gonadectomised mice). A positive result in any of these mice is taken as positive evidence of the existence of pregnancy.

(b) Preliminary Investigations.

Preliminary investigations were carried out on a herd of 8 Shetland ponies and one Icelandic pony. Unfortunately only the approximate date of service of 5 of them was known. Of the remainder 3 had been served on a known date, and the other had not been mated.

Urine was obtained from these animals by catheterisation. In some animals it was found easier to collect urine than from others, and this did not always seem to be related to the amount of urine in the bladder, as this difficulty was experienced with the same animals over a period of about 9 months, but rather to the consistency of/
of/

of the urine. The urine of one mare in particular was almost without exception of a turbid and very tenacious mucinous character and this animal always proved difficult to collect urine from. All these animals were at grass, the only addition to their diet being some hay during the winter months. The normal urine of the horse, according to Smith (71) is always turbid, but this is certainly not so in the Shetland pony. It is just as common to get urine of a clear watery consistency and such urine is easily drawn off. The colour varied from a yellow to an almost clear fluid. The amount obtainable in most cases was small and the Shetland pony is not regarded as so satisfactory an animal for the collection of large amounts of urine as larger animals. There is no reason, however, why Shetland ponies should not be used as a source of the alpha hormone. They are more economical to keep than the bigger breeds and pass off sufficient urine in a day to yield large quantities of this hormone. The difficulties of collection could be overcome by using only those animals which yield their urine readily or by the adoption of a suitable type of urine bag which is attached to the animals.

If/

If carefully and gradually applied, this latter method would, in time, yield the maximum amount of urine. Zondek emphasises the importance of the mare as a source of this hormone. He states that a pregnant mare can produce as much hormone as nearly 1,500 patients from a maternity clinic.

Four immature mice were used for these preliminary tests, each receiving respectively 0.2 c.c., 0.25 c.c., 0.3 c.c. and 0.5 c.c. twice daily for 3 days. Smears were made as usual on the 5th day.

Urine from Shetland pony C gave a positive reaction on all four mice. This indicates a concentration of at least 800 m.u. per litre. At the time this reaction was regarded as positive evidence of pregnancy, but in view of the knowledge we now possess of the excretion of this hormone during the oestrous cycle in amounts up to this figure, it would be incorrect to regard this as a positive result. The demonstration of a concentration of 800 m.u. per litre, might, outside the sexual season, be regarded as evidence of pregnancy, but the possibility of the occurrence of oestrus at other times of the year, though rare, must not be overlooked/

overlooked. Moreover, different breeds do not all commence the sexual cycle at the same time of the year, apart from individual differences and from those which occur in different countries, as for example in India. For the easy application of the test it is essential that a method should be capable of being carried out in a uniform manner throughout the whole year.

Specimens of urine from Shetland ponies B, D, E, F, G, H and I all gave 800 m.u. per litre. Mare A, which had not been served gave negative reactions on all tests. That the occurrence of this hormone was not transient was demonstrated by its appearance in successive samples; for example, mare H gave positive results with urine taken on two successive days and again 14 days later.

Vaginal smears were made daily commencing on the first day of injection of pregnant urine. On the first two days the vaginal contents were typically negative, consisting of nucleated epithelial cells and leucocytes. The response to the presence of the alpha hormone is gradual and is indicated primarily by an alteration in the relative proportions of epithelial cells and leucocytes. All the/

the stages that appear in the normal unmated mouse can be seen. The leucocytes gradually become fewer in number until the pro-oestrus condition is reached. Rarely complete cornification occurs on the 3rd day, sometimes on the 4th, and usually on the 5th. In some cases it was delayed until the 6th day.

Table I.

SPECIMEN OF URINE	MICE INJECTED					DAY OF TEST					RESULT
						1ST	2ND	3RD	4TH	5TH	
PF 50	I	02	CC	X 6	EL	EL	EL	Ecc	CC	POSITIVE	
	II	025	CC	X 6	EL	EL	E	Ecc	CC		
	III	03	CC	X 6	EL	EL	E	Ecc	CC		
	IV	05	CC	X 6	EL	EL	E	Ecc	CC		
PF 54	I	02	CC	X 6	EL	EL	EL	EL	EL	NEGATIVE	
	II	025	CC	X 6	EL	EL	EL	EL	EL		
	III	03	CC	X 6	EL	EL	EL	EL	EL		
	IV	05	CC	X 6	EL	EL	EL	EL	EL		

EL= NUCLEATED EPITHELIAL CELLS AND LEUCOCYTES.

E = NUCLEATED EPITHELIAL CELLS.

Ecc = NUCLEATED EPITHELIAL CELLS + A FEW CORNIFIED CELLS.

CC= COMPLETE CORNIFICATION.

It was decided, in view of these suggestive results, that an extensive trial should be carried out to determine whether a reliable method of diagnosing pregnancy could be elaborated and concurrently/

concurrently to follow the excretion of the alpha hormone in the urine throughout the entire period of pregnancy and also to discover the earliest time at which a positive determination of pregnancy could be made.

(c) Excretion in the Pregnant Mare.

Urine was examined from mares in the early stages of pregnancy to determine the time at which the alpha hormone appeared in a concentration sufficient to indicate that conception had occurred. Immature and castrated mice were used in view of the possibility of there being present in the urine a hormone derived from the anterior lobe of the pituitary, the rho one factor, which is regarded as providing the necessary stimulus for the maturation of the Graafian follicles. Further, this oestrogenic factor stimulates the ovary to produce the alpha hormone which in turn induces the changes in the uterus and vagina typical of the normal oestrous cycle. It might quite well be the case that the rho one factor appears in the urine at an earlier stage than the alpha factor. If this were so, the early diagnosis of pregnancy (before the 60th day) could be based on this hormone. The question/

question of the occurrence of anterior pituitary hormone or hormones in the urine has already been dealt with. It is of interest to note here that pregnancy can be diagnosed in women as early as about the 10th day after conception by the Zondek-Aschheim test. This is based on an anterior pituitary hormone, rho two, but the alpha hormone, states Zondek, is only doubtfully present during the first two months.

Specimens of urine taken from mares less than a month after service did not show any sign of the alpha factor. It is not quite correct, however, to state that no alpha was found as with the injections used, the lowest concentration that could be demonstrated was 300 m.u. per litre. As far as the practical application of the test is concerned, amounts below this figure can be neglected. In order that the early period of pregnancy could be followed closely, regular and frequent specimens of urine were collected from a Clydesdale mare and two Shetland ponies. The results obtained from these animals, together with those from various mares at different intervals after mating, indicate the time of the appearance of the alpha hormone in the urine in detectable amounts and also its gradual increase.

The/

The alpha hormone first appeared in the urine of the Clydesdale mare 47 days after mating. The concentration found was 833 m.u. per litre, but this amount varied from 500-800 m.u. up to the 60th day after mating with an occasional negative result. Specimens of urine taken after this time were uniformly negative until the 89th day of gestation, when 2000 units were found.

Table II.

APPEARANCE OF THE ALPHA HORMONE IN THE URINE IN A DIAGNOSTIC CONCENTRATION IN THE EARLY STAGES OF PREGNANCY.		
MARE	Nº OF DAYS SINCE LAST SERVICE	Nº OF M. U. PER LITRE.
SHETLAND PONY	65	2,400
HUNTER	76	2,000
"	79	1,000
"	82	2,500

A Shetland pony mare gave negative results up to the 60th day of pregnancy, but on the 65th day 2400 m.u. were found. Three mares Pf.247,249 and 248 on the 72nd, 90th and 92nd days of pregnancy gave a concentration of 600-800 units. Further specimens from these mares were tested on the 89th, 93rd/

93rd and 102nd days of pregnancy and gave respectively 1600, 2000 and 1000 m.u. per litre. These results emphasise the individual variation in the excretion of the alpha hormone.

The inference to be drawn from these results, is that the alpha hormone is very variable in the time of its appearance in the urine in the early stages of pregnancy and that the amount excreted may vary within wide limits. The earliest time at which a positive result was obtained was the 65th day, but this will probably be reduced when a larger number of mares in the early stages of pregnancy are examined.

On the 76th day of pregnancy as much as 2000 m.u. per litre of the alpha hormone were found. This amount represents a marked increase over that found in the normal unmated animal or in pathological conditions such as cystic ovaries. In the latter condition the concentration of alpha never, in those cases where it was found to be present in the urine, exceeded the amount present in the normal unmated animal during the oestrous cycle. It is not known yet whether nymphomaniac mares excrete this hormone in greater amount than that found/

found so far, or whether there is any other pathological condition which will cause this hormone to be present in the urine in relatively high concentration. It would seem unlikely, having in mind the nature of the nymphomaniac, that a concentration much greater than that found during the oestrous cycle would be found.

The concentration of 2,000 m.u. per litre leaves quite a large margin of safety and it is possible to base a diagnosis on lesser amounts. The largest amount found outside pregnancy was 833 m.u. per litre and probably the discovery of a concentration greater than 1,000 m.u. per litre is sufficient for diagnostic purposes. With small differences such as this, however, the possibility of error is greater and it is highly desirable that the urine of mares which give a result indicating a concentration of 1,000 m.u. per litre or less be subjected to re-test after a short interval. It would not appear to be necessary to carry out further tests when the concentration exceeds 1,000 m.u. per litre.

Table III /

Table III.

MARE	Nº OF DAYS PREGNANT ;	Nº OF M. U. PER LITRE.
PF 245	86	1600
PF 246	90	2,500
PF 250	95	3,500
PF 211	104	4,100
PF 45	130	25,000
PF 259	162	41,600
PF 61	165	111,100
PF 96	187	166,600
PF 265	248	222,200

As would be expected the concentration of the alpha hormone in the urine increases as pregnancy advances. By the 95th day the excretion has reached 2,500 m.u. per litre and on the 104th day over 4,000 units. Thereafter it steadily increased until the 187th day when it amounted to over 160,000 units. The maximum concentration found was over 200,000 m.u. per litre.

Considerable variation in the amount of this hormone excreted in the urine was noted in the case of mares which had been pregnant for approximately the/

the same length of time. While this may partly be explained by the difference in response of the test animals, it would also seem to be due to a variation in the amount excreted by individual mares. An example of this variation is shown in Tables IV and V.

Table IV.

MARE	Nº OF DAYS PREGNANT	Nº OF M. U. PER LITRE URINE
S (CLYDESDALE)	270	166,600
	282	166,600
	286	133,300
	287	133,300
	288	133,300
	316	166,600

There was a marked similarity between the Shetland ponies and the larger breeds of equines in respect to alpha secretion. Shetland pony mare B, at least 127 days after mating, contained 6,600 m.u. per litre of urine. Six specimens of urine were taken from this mare during pregnancy and an increase in the amount of hormone secreted was noted/

noted as pregnancy progressed. The amount of hormone found from the 127th-160th day of pregnancy was from 6,600-11,100 m.u. per litre, from the 160th-200th day 11,000-100,000 units, and from the 200th day up to 160,000 units.

Table V.

MARE	Nº OF DAYS PREGNANT	Nº OF M. U. PER LITRE
E	106	800†
(SHETLAND PONY)	185	111,100
	205	66,600
	233	133,300
	266	133,300
	273	133,300

† AS THIS WAS ONE OF THE FIRST TESTS MADE NO ESTIMATION OF CONCENTRATION BEYOND THAT INDICATED BY ORDINARY TEST, WAS CARRIED OUT.

It will be seen that the amount of the alpha hormone in the urine of the pregnant Shetland pony is much the same as that found in the bigger breeds, such as the Clydesdale and the thoroughbred. A total of 71 tests were carried out on these ponies (excluding those made during the sexual season). Mare A which had not been mated, served as a control and gave uniformly negative results over all tests.

Table VI. /

Table VI.

MARE	PREGNANT OR NON-PREGNANT	RESULT OF TEST
A	NON - PREGNANT	NEGATIVE
B	PREGNANT	POSITIVE
C	" "	" "
D	" "	" "
E	" "	" "
F	" "	" "
G	" "	" "
H	" "	" "
I	" "	" "

Results.

In all a total number of 397 tests were carried out on animals consisting of Shetland ponies, Clydesdales, hunters, "country-bred" Indian mares and an Icelandic pony. In several instances where the first result was inconclusive, second specimens of urine were tested, while a large number of specimens from a Clydesdale mare and from 9 Shetland ponies were tested throughout the whole period of pregnancy. A total of 64 tests were carried out on Shetland ponies. Of these, 34 were positive/

positive and 30 negative. The negative results were given by non-pregnant mares and by other mares in the early stages of pregnancy, before the appearance of the hormone in the urine. The non-pregnant animals gave negative results in every instance and positive results were obtained from the pregnant mares with every specimen, except one taken from mare F. Thus there is no error in the non-pregnant mares and an error of 2.27 per cent. for the pregnant mares. As all the subsequent specimens taken from this mare were positive a positive diagnosis was made. This figure does not, therefore, represent a real error but merely the absence of hormone in one (the first) specimen of urine taken from this mare. Special mention of this sample is made here because it was taken on the 120th day of pregnancy.

Of the other breeds of horses, 106 were examined, and of all the tests carried out, 218 were positive and 115 negative. The negative results include those taken in the early stages of pregnancy when it was not possible to give a diagnosis. Up to date 63 cases are known to be pregnant or non-pregnant or to have foaled or aborted/

aborted, in each case proving the case to be correct.

In three cases the biological diagnosis did not coincide with the clinical. The importance of these latter results will be emphasised more fully later; here it is sufficient to draw attention to the fact that two of these results were obtained in the first week or two of the test and were not based on a quantitative estimation of the hormone, which is now regarded as essential.

(d) Disappearance of the Alpha Hormone from the Urine after Parturition.

Immediately before parturition the concentration of the alpha hormone was 166,000 m.u. per litre. Thirty-five hours after foaling it had fallen to 800 units and after sixty-one hours it was less than 300 units.

(e) The Chemistry of the Alpha Hormone in the Urine of the Pregnant Mare.

The property possessed by the alpha hormone of retaining its activity over long periods was demonstrated. Urine which had been collected some months previously and to which toluol had been added, still retained a large quantity of hormone/

hormone. Also specimens sent from India (where some five weeks elapsed between taking the sample and carrying out the test) were found to contain the alpha hormone in a concentration equivalent to that obtained from mares in this country at a similar period of gestation.

Its resistance to heat was demonstrated by experiments involving the concentration of the urine. Evaporation by heat had no effect on the activity of the hormone.

In order that an examination of the urine might be made for the anterior pituitary factors the removal of alpha by extraction with ether (alpha in the urine of pregnant women is soluble in ether) was attempted. The residual urine, however, after extraction was found to be active in castrated mice, and it was concluded that the alpha hormone in the urine of the pregnant mare was not soluble in ether. The reason for its non-solubility in ether in the case of the mare and its solubility in ether in the case of the pregnant woman may be due to differences in the composition of mares' urine. This finding is in agreement with that of Zondek who was unable to extract this hormone with ether. On the other hand he found that/

that after treatment of the urine with ether the hormonal content becomes greater, up to 40 per cent. He concluded from this that ether had removed some inhibitory substance from the urine. As Zondek found an average of 100,000 units and as over 160,000 units have been demonstrated in this paper, the presence of an inhibitory substance in the urine, capable of being removed by ether, would not seem to have been proved.

(f) Excretion in the Non-pregnant Mare during the Oestrous Cycle.

Urine was collected from two Shetland ponies, in one case over three cycles and in the other throughout one cycle. Specimens were thus obtained at all periods of the sexual cycle. The actual time of heat was determined by a stallion. The mares were "tested" by the stallion daily throughout the period of investigation. The stage of heat was determined by the behaviour of the mare towards the stallion, her stationary position, not showing any inclination to move away when mounted by the stallion, the raising of the tail, being taken as evidence of heat. The mounting of the mare/

mare by the stallion is not in itself sufficient evidence of heat, nor are the clinical signs of oestrus, the spasmodic erection of the clitoris, the frequent passage of urine, since these may be in evidence before the mare is willing to accept the horse.

In the case of the Clydesdale mare it was only possible to determine her condition when visited by a stallion which was on circuit in the district. This proved very unsatisfactory as it was not possible to determine the various stages with even an approximate degree of accuracy; indeed the "foal" heat was missed entirely. In following the excretion of the alpha hormone in the urine during the oestrous cycle, it is essential that the exact stage of the cycle at which the specimen of urine was obtained, is known. The only efficient way of doing this is to keep a stallion with which the mare can be "tested". Probably a vasectomised animal would be more efficient as the danger of successful copulation taking place would be obviated. The method adopted here was to allow the Shetland stallion to mount the mares, but he was pulled off before service could be accomplished, sufficient/

sufficient time, however, being allowed to elapse after mounting to determine if the mare was in heat. That this is a successful method of determining heat without pregnancy ensuing is shown by the fact that one of the Shetland mares which was "tested" at every heat period in this manner did not become pregnant until the stallion was allowed to serve her normally.

Specimens of urine taken from the Shetland ponies at all stages of the oestrous cycle were uniformly negative, i.e. nothing above 300 m.u. per litre of the alpha hormone was found. It was expected that it would be present in the urine during the stage of oestrus, but this was not so, in the Shetland ponies at least. Results obtained from the Clydesdale mare are of lesser value, as it was only possible to determine one period of heat from the day on which service took place.

The first occurrence of heat after foaling tends to be irregular. The usual time is 11 days after parturition, but in some cases it does not occur until 17 days after parturition. Mare S was not in heat 21 days after foaling when tested by the stallion. However, on the 14th day the presence of alpha was noted in the urine. Alpha continued/

continued to be excreted for 12 days in amounts varying from 550-833 m.u. per litre of urine. The mare was served eventually 41 days after foaling. If we regard the whole cycle as taking about 21 days, the mare would have been on heat approximately 20 days after parturition. Thus it would appear that alpha is excreted in the urine during the pro-oestrous, oestrous and possibly metoestrous stages, but from this information it is impossible to state definitely at what period or periods of the oestrous cycle the alpha hormone is excreted.

More definite information is available from the second heat period. On the day of service 300 m.u. were found, on the following day less than 300, on the 5th day 550 units were present. On the evening of the 3rd day, however, less than 300 units were found to be present. The most striking difference between this elimination of alpha and the previous one is the short space of time over which it occurred, 2 days, or at the most 5 days (a specimen of urine taken on the 38th day after foaling was negative), compared with 12 days. Here it seems to be more definitely restricted to the actual period of heat. The urine taken in the inter-oestral/

inter-oestral periods gave negative results with the lowest concentration used, i.e. 300 m.u. per litre. Concentration of urine by evaporation, however, revealed amounts equivalent to 160 m.u. per litre. This amount was found over a period of 15 days, up to 4 days before service. A similar amount was found in the early stages of pregnancy. It may be that this is the amount found normally in the non-pregnant animal. The possibility of the presence of alpha in food will have to be considered in this connection.

Further work will have to be done on the oestrous cycle and the alpha hormone, but, to summarise the above results, the alpha hormone was found in the urine in a concentration of 300-800 m.u. per litre during oestrus and 160 m.u. during the inter-oestral period.

(g) Excretion in the Gelding.

A number of specimens of urine from geldings were examined for this hormone. The urine was obtained from a knackery and only small quantities were available for investigation. Negative results were obtained in every case. As it was not/

not possible to carry out tests with concentrated urine, owing to the small amounts obtained, it may be that the hormone does occur in such urine, but in amounts less than 300 units.

(h) Excretion in Nymphomaniacs.

It seems that nymphomania in the mare may be associated with the excretion of the alpha hormone in the urine. A concentration very similar to that of the non-pregnant mare during heat was found in the urine of nymphomaniac mares.

Specimens were examined from three cases. Mare No. 1 showed signs of continuous oestrus, but no trace of the alpha hormone was found (lowest concentration used, 300 units per litre). Mares Nos. 2 and 3 who were also showing signs of continuous oestrus contained in their urine 800 and 300 m.u. per litre respectively of this hormone. In view of the more intense nature of the symptoms a higher concentration might have been expected. These symptoms, however, which are purely sexual do not differ greatly from those of normal oestrus in the mare. Temperamentally there is a marked difference. What effect this hormone/

hormone may have on the temperament of animal it is difficult to say; certainly even in the normal mare there is increased excitability. On the other hand during pregnancy, when immense amounts of this hormone are found, the behaviour of the animal is normal. It is possible that there is a greater production of hormone in this condition than in the non-pregnant mare during oestrus, but that it is utilised by the animal. It is only the surplus that appears in the urine.

Table VII.

NYMPHOMANIAC MARES	RESULT OF TEST	Nº OF M. U. PER LITRE URINE
Nº 1	NEGATIVE	—
Nº 2	POSITIVE	800
Nº 3	" "	300

The anterior lobe of the pituitary is now known to exert a controlling influence over the ovary, by means of two hormones, rho one, the follicle maturing factor, and rho two, the factor responsible for luteinisation. It seems reasonable, therefore, to postulate that in some cases the condition arises from aberrant functioning of the anterior pituitary. The/

The stimulus which causes the anterior pituitary to function at puberty or at the beginning of a new sexual season, or periodically throughout the year, as the case may be, is unknown. The stimulus, which is normally confined to these periods, may in cases of nymphomania, arise independently of them. This starts the train of action, the anterior pituitary under the influence of this unknown stimulus elaborates its hormonal secretion and the ovary responds by the production of the alpha hormone. Maturation of the follicles takes place and eventually the signs of oestrus appear. The unknown stimulus may be periodic in its action or it may be continuous, thus explaining the periodic or the continuous nature of the symptoms.

The continuation of symptoms after removal of the diseased ovaries, whether this be the origin of the condition or the medium through which the effects are produced, may be related to the length of time that the condition has been present. The expression of symptoms here is the reflection of an internal stimulus. The animal in which the disease process has been at work for/

for some time has become accustomed to the behaviour arising from it, and as a result, when the stimulus is removed, no alteration takes place. It has in fact developed a habit which is now so ingrained that it can continue in the absence of the organ responsible for the disease.

The usual reason advanced for the occurrence of ovarian cysts is that for some reason or other the wall of the follicle has become so thickened that rupture cannot take place. The follicular fluid continues to be secreted and the follicle may reach a considerable size. In the absence of rupture the anterior pituitary continues to produce the follicle maturing hormone, and the mare shows signs of continuous oestrus. The periodic appearance of oestrus does not necessarily mean that rupture has taken place, but merely that the original stimulus is periodic in the appearance.

VIII./

VIII. EXAMINATION OF MILK DURING THE OESTROUS CYCLE
FOR THE ALPHA HORMONE.

Specimens of milk were obtained from a Shetland pony during the oestrous, metoestrous and dioestrous periods of the sexual cycle. The heat period in this particular case lasted 5 days, and milk taken on the last 2 days gave no reaction when injected into ovariectomised animals (the least amount demonstrable by the test was 300 m.u. per litre). Similarly, no hormone was found during metoestrus and dioestrus. It was not possible to obtain sufficiently large quantities of milk to examine it for a hormonal concentration of less than 300 m.u.

IX. /

IX. DISCUSSION

As the alpha hormone occurs in the non-pregnant animal during the oestrous cycle and in nymphomaniac mares, it is necessary that a concentration greater than that found outside pregnancy should be demonstrated before a positive biological diagnosis can be made. It was customary in the early days of the test to notify those who sent in specimens of urine that a positive result had been obtained from the test whenever positive reactions occurred in the test animal, which had been injected with normal, undiluted urine. The maximum concentration available in this manner is 833 m.u. per litre and as equivalent amounts are now known to occur during the oestrous cycle and in cases of cystic ovaries, such a concentration is not regarded as indicative of pregnancy. It was emphasised that the results obtained at this stage were not to be regarded as conclusive as the test was still in its experimental stages. Later no positive notification was given unless the concentration exceeded 1,000 m.u. per litre.

Two/

Two "apparent" errors arose in this way. Mares PF.59 and 62, 154 and 178 days after service, respectively, were found to give in their urine a hormonal concentration of 830 units. No retest was made and the mares subsequently proved to be not in foal. The necessity of controlling such results is made evident by the case of mare PF.50, 179 days after service. This mare likewise gave a concentration of 830 units, but was negative for a concentration of 25,000 units (a concentration that might have been expected at this period). This case was controlled by a second specimen taken 280 days after service, when a negative result was obtained. The diagnosis that this mare was not pregnant proved to be correct.

Why a mare should at this period, well after the end of the sexual season, excrete the alpha hormone in this amount in the urine is obscure. It is possible that it represents the occurrence of heat at a time outside the normal sexual season, a somewhat rare occurrence. The fact that this result was obtained from more than one animal would seem to eliminate this possibility. The question of abortion does not appear to be of great significance, as if mare PF.50 had been pregnant at the/

the time of testing the first specimen, a much greater amount of alpha would have been found. Useful information would be obtained from the examination of specimens of urine taken at frequent intervals throughout the year from non-pregnant mares and from mares which had been mated but had not conceived. By this means it would be possible to determine the extent of alpha excretion in the non-pregnant mare during the normally quiescent periods between breeding seasons. Zondek mentions that Kust and Grawert found the alpha hormone in the urine of the mare, not only during pregnancy, but also in the urine of mares which had been mated but had not conceived. It may be that the act of coitus provides the necessary stimulus for the elaboration of alpha by the ovary, irrespective of whether conception has taken place, and that following a sterile coitus the excretion of this hormone occurs in a manner similar to its excretion in the early stages of pregnancy, but in a much lesser degree. This apparently continues for a period of at least 179 days. In fact, the period of elimination of alpha in the urine of the non-pregnant animal which has been mated but which has/

has not conceived may be regarded as a period of pseudo-pregnancy.

A positive result was obtained from a mare which subsequently proved to be non-pregnant. Since a concentration of 16,600 m.u. per litre was found, this result was a little difficult to understand. The most obvious explanation is that abortion had taken place after collection of the urine. The mare was tested 96 days after service. It is possible that the mare was at grass and the abortion passed unnoticed, but no definite information was available. Another case not included in the results because of lack of definite information concerning it, gave a concentration of 166,600 units per litre. The owner himself suggested that as the mare had been at grass it was possible that abortion had occurred unnoticed by anyone.

The information afforded by the test has so far proved of such value that it may be safely assumed that these mares were actually pregnant at the time of the test, but that abortion subsequently occurred. In view of the very large number of mares which fail to yield a foal every year, it would/

would be of great interest to know whether this is due to failure to conceive or to interruption of pregnancy at a later date. It is in such cases that this test should prove of value. The fact that a positive test on a certain date was followed by a negative test on a subsequent date would indicate that interruption of pregnancy had occurred.

It has been stated that before a positive diagnosis of pregnancy can be given the amount of alpha must exceed that normally found in the non-pregnant animal. In some cases this may not be necessary, particularly where the mares are closely watched during the sexual season and their condition (recurrence of heat) is known. A concentration of 800 m.u. per litre should be regarded as significant if combined with a knowledge of absence of oestrus in previously mated animals. However, in this connection the question of pseudo-pregnancy will have to be borne in mind, and a confirmatory test at a later date would be advisable.

Three mares, PF. 247, PF. 249 and PF. 248 on the 72nd, 90th and 92nd days of pregnancy gave from 600-800 units. Second specimens were tested on the 89th, 93rd and 102nd days of pregnancy respectively, and all gave positive results. These results/

results emphasise the individual variation which occurs in the excretion of this hormone and indicate that while in some cases pregnancy may be diagnosed as early as the 65th day and perhaps earlier, it is not possible to lay down a hard and fast rule.

Frank mentions the case of a woman who gave a positive result for the alpha factor some days after birth, when the membranes were supposed to have been completely removed. On examination this was found to be not so. The complete removal of the membranes led to the disappearance of the hormone from the urine. Specimens of urine have been received at the Human Pregnancy Diagnosis Station here, asking if abortion was complete. The gonadotrophic hormone may, however, occur in the urine as long as 9 days after complete abortion, and it is not until this period has elapsed that the test would be of value. In the mare, on the other hand, the alpha hormone disappears relatively quickly - within 3 days - and the test may eventually prove of value in cases of suspected retention of the placenta. Sterility in the lower animals is frequently associated with an infected condition of the genital tract, probably, in many cases dating from/

from the time of parturition. One of the causes of infection at this time may be the failure of the animal to rid itself completely or sufficiently quickly of the membranes. The application of a biological test to valuable pedigree mares as a routine measure, say a few days after foaling might (if the placental connection was still functional) afford exceedingly useful information.

Errors may arise from the test animals themselves. The presence of accessory ovaries in mice is rare. Parkes who dissected some thousands of mice found only two with accessory ovaries, and in these the supplementary body was contained within the same capsule as the normal ovary. Error from this source may therefore be eliminated as it is the custom when ovariectomising animals to remove the ovarian capsule and the proximate extremity of the uterine tubes along with the ovaries. The regeneration of ovarian tissue must, however, be considered. Several workers have shown that regeneration occurs after complete removal of both ovaries, and in view of this, the appearance of oestrus in one of the test animals is probably not sufficient to indicate a positive result. A specimen/

specimen of urine which gives a positive result on only one test animal should therefore be subjected to a re-test.

It is known that cornification of the vaginal epithelium is not absolutely specific for oestrus. Evans found that vitamin A deficiency produced cornification even in the ovariectomised animal. The diet of all test animals should, therefore, be as complete as possible.

The question of the time at which test mice should be disposed of after they have been in use for some time will have to be considered in view of the possibility of error arising from their frequent use. In this investigation mice were not kept for longer than 4 months.

The gonadotrophic action of the anterior pituitary body in the mare does not seem to differ in any way from that of other animals. The two factors, one stimulating follicular maturation and the other causing luteinisation, are both present in the mare. The difference is that, while in woman they both occur in the urine and the blood, in the mare the luteinisation factor is absent from the urine/

urine. Zondek found much smaller amounts of both factors in the blood of pregnant mares, than in women, which indicates that much less is being produced. In the pregnant woman these hormones occur in the placenta. Whether this is a source or merely a reservoir is still an open question. The placenta in the mare is the most primitive type known and it may be that the difference in these hormones in the blood of woman and of the mare can be explained by the diverse types of placentation. No investigation has, as yet, been made of the placenta of the mare for these hormones. The amount of the maturation factor of the anterior pituitary in the urine is quite small and the absence of the luteinisation factor may be due to it being used up completely by the foetus.

X. /

X. SUMMARY.

1. The test is based on the presence of the alpha hormone (oestrin) in the urine of the pregnant mare.

2. A total of 397 tests were carried out on 115 equines. Positive results were obtained in 282 tests and negative results in the remainder.

3. The number of mares that were notified as being pregnant was 37 and positive results were obtained in 38 cases, one mare proving to be not in foal. The error in pregnant mares is therefore 2.7 per cent.

4. Twenty-eight non-pregnant mares were examined and the test was correct in each case.

5. A gradual increase in the concentration of the hormone in the urine throughout the period of pregnancy was noted. 800 m.u. per litre was found on the 47th day of pregnancy and towards the end of the period over 166,000 m.u. per litre.

6. /

6. The diagnosis of pregnancy was based on a quantitative estimation of the hormone. 1,000 m.u. per litre was regarded as the minimum amount on which a positive diagnosis could be made.

7. The earliest time at which it was possible to diagnose pregnancy by this method was at the 65th day after mating.

8. Thirty-five hours after foaling the concentration of the hormone had fallen to 800 m.u. per litre, and after sixty-one hours it was less than 300.

9. The alpha hormone was found in the urine in a concentration of 300-800 m.u. per litre during oestrus and of 160 units during the inter-oestral and anoestral periods.

10. Similar amounts to that found during oestrus were discovered in the urine of two nymphomaniac mares.

11. Milk from a lactating mare failed to reveal signs/

signs of this hormone.

12. The alpha hormone in the urine of mares was not soluble in ether.

13. No sign of anterior pituitary hormones, rho one and rho two, was noted and therefore it was concluded that it was not possible to diagnose early pregnancy in the mare by the presence of the rho one factor in the urine.

XI. /

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XII. /

XII. BIBLIOGRAPHY

1. Aitken, W.A. 1927. J. Amer. Vet. Assoc. 70, N.S. 23, 4, 481.
2. Allan, Dickens, Dodds and Howitt. A study of the oestrous producing hormone; its preparation and standardisation in a water soluble form. 1928. Biochem. J. 22.
3. Allen, quoted by Parkes. The Internal Secretions of the Ovary, p. 44.
4. Allen, E. and Doisy, E.A. An ovarian hormone. Preliminary report on its localisation, extraction and partial purification and action in test animals. 1923. J. Amer. Med. Ass. 81, 819.
5. Allen and Doisy, quoted by Parkes. The Internal Secretions of the Ovary, p. 110.
6. Aschheim and Zondek. Die Schwangerschafts diagnose aus dem Harn durch Nachweis des Hypophysenvorderlappenhormons. 1928. Klin. Wschr. 7.
7. Assheton, quoted by Marshall. The physiology of Reproduction, p. 430.
8. Binz, quoted by Frank. The Female Sex Hormone, p.112.
9. /

9. Bucura, quoted by Parkes. The Internal Secretion of the Ovary, p. 83.
10. Bugbee, E.P. and Simond, A.E. Standardisation of ovarian hormone. 1926. Endocrin. 10, 191.
11. Cole, H.H. and Hart, G.H. The potency of blood serum in mares in progressive stages of pregnancy in effecting the sexual maturity of the immature rat. 1930. Amer. J. Physiol. 93, 57.
12. Idem. Sex hormones in the blood serum of mares. II. The sera from 22nd day of pregnancy to the first heat period post partum. Ibid. 1930. 94, 597.
13. Courrier, quoted by Frank. The Female Sex Hormone, p. 102.
14. Coward, K.H. and Burn, J.H. The variation in the unit of the oestrus-producing hormone. 1927. J. Physiol. 63, 270.
15. Crew, F.A.E. Proc. Roy. Soc. 1923, B.95.
16. Idem. 1931. Vet. Rec. 18, 19.
17. Dickens, F., Dodds, E.C. and Brinkwater, D.J.T. 1927. Lancet, 1, 1015.
18. Dohrn, Faure, Poll and Blotevogel. Tokokinine, Stoffe mit sexualhormonartiger Wirkung aus Pflanzenzellen. 1926, Med. Klinik, 22.

19. Dohrn, M. and Faure, W. Excretion of the female sex hormone. 1928. Klin. Wschr. 7, 943.
20. Evans and Long. The effect of the anterior lobe administered intra-peritoneally upon growth, maturity and oestrous cycles of the rat. 1921. Anat. Rec. 21, 62.
21. Ewart, Cossar. Studies on the Development of the Horse. 1915. Trans. Roy. Soc. Edin. 51.
21. b. Idem. A Critical Period in the Development of the Horse. 1897. Adam and Charles Black. Lond.
22. a. Frank, R.T. The Female Sex Hormone, Thomas, Baltimore. pp.63,64.
- b. Ibid. p. 73.
- c. Ibid. pp.117-119.
- d. Ibid. pp.189-190.
- e. Ibid. p. 252.
- f. Ibid. p. 194.
- g. Ibid. pp. 129-146.
- h. Ibid. p. 120.
- i. Ibid. p. 127.
- j. Ibid. p. 113.
- k. Ibid. p. 203.
23. Frank and Gustavson The Female Sex Hormone, p.103. quoted by Frank.

24. Frank, Frank, Gustavson and Weyerts, quoted by Frank The Female Sex Hormone, p. 113.
25. Frank and Goldberger quoted by Frank. The Female Sex Hormone, p. 89.
26. Frank and Rosenbloom. quoted in The Female Sex Hormone, p. 63.
27. Frank, A.H. Missouri Agri. Stat. Bull. 150.
28. Fels, E. Presence of ovarian hormone in the blood of normal and pregnant women. 1926. Klin. Wschr. 5, 2349.
29. Fellner, in The Internal Secretions of the Ovary, p. 84.
30. Idem. in The Female Sex Hormone, p. 112.
31. Halliburton and McDowall Handb. of Physiol. 1930.
32. Herrman and Fraenkel in The Internal Secretions of the Ovary, p. 84.
33. Hirsch, quoted by Frank in The Female Sex Hormone, p.113.
34. Hirsch, Brunsthormon im Blute des Mannes. 1928. Klin. Wschr. 7.
35. Hisaw and Meyer. The oestrus-producing hormone in the urine of pregnant cows. 1929. Proc. Soc. Exp. Biol. Med. 26, 585.

36. Iscovesco The Female Sex Hormone, p. 63.
37. Jentzer and Beuttner, quoted by Parkes in The
Internal Secretions of the
Ovary, p. 83.
38. Jordan, C.N. and The effect of light upon the
Doisy, E.A. follicular hormone. 1926.
Proc. Soc. Exper. Biol. Med.
24, 216.
39. Kahnt, L.C. and The vaginal smear method of
Doisy, E.A. assay of the ovarian hormone.
1928. Endocrin. 12, 760.
40. Küst. Die Festullung der Trachigkeit
der Stute durch den Nachweis
des Ovarialhormone im Harn.
1931. Dtsch. Tierarztl. Wschr.
39, 33.
41. Laquer, E. Uber weibliche (Sexual-)
Hormone im bes Eichung. 1928.
Physiol. Path. Therap. 2, 133.
42. Laquer, E., Hart, Uber weibliches Sexual Hormon
P.C. and de Jongh, Menformon. 1926. Dtsch.
S.E. med. Wschr. 52.
43. Laquer, E. and de A female (sexual) hormone.
Jongh, S.E. 1928. J. Amer. med. Ass. 91.
44. Laquer, E. quoted by Frank in The Female
Sex Hormone, p. 113.
45. Lipschütz, A. and The presence of oestrin in
Veshnjokov, S. the urine of the pregnant cow.
1930. 220, 456.
46. Loewe, S. Nomenclature of Sexual Hormone.
1926. Dtsch. med. Wschr. 52, 1685.

47. Loewe, quoted by Parkes in The Internal Secretions of the Ovary, p. 108.
48. Loewe, S. and Lange, F. Der Gehalt des Frauenharnes an brunsterzeugen den Stoffe in Abhangigkeit von ovariellen Zyklus. 1926. Klin. Wschr. 5, 1038.
49. Idem. Uber weibliche Sexual Hormon. IX. Das "Zahlverfahren" zur biologischen Titration Brunst-hormonhaltiger Stoffe seine Fehlerquellen und deren Vermeidung. 1926. Dtsch. med. Wschr. 52, 1286.
50. Loewe, Lange and Spohr Uber weibliche Sexualhormone (Thelytropine) XII. Brunsterzeugende Stoffe (Thelykinine) als Erzeugnisse des Pflanzenreiches. 1927. Biochem. Z. 180.
51. Loewe, Voss, Lange and Wahner. Sexualhormonbefunde im mannlichen Harn. 1928. Klin. Wschr. 7.
52. Long and Evans. The oestrous cycle in the rat and its associated phenomena. 1922. Mems. Univ. Calif. 6.
53. Ludwig, F. and Ries, J.V. Actino-activity of oestrin. 1931. Z. Gynak. 17th Jan. p. 137.
54. Marrian, G.F. Chemistry of oestrin. 1929. Biochem. J. 5.
- 55.a. Marshall, F.H.A. The Physiology of Reproduction, p. 46.

- 55.b. Marshall, F.H.A. The Physiology of Reproduction,
pp. 129-131.
- c. ibid. pp. 74 et seq.
- d. ibid. pp. 427-432.
- e. ibid. p. 444.
- f. ibid. p. 490 et seq.
56. Marshall, F.H.A. quoted by Parkes in The
and Jolly, W.A. Internal Secretions of the
Ovary, p. 83.
57. Idem. quoted by Frank, The Female
Sex Hormone.
58. Mazer and Hoffman, quoted by Frank, The Female
Sex Hormone, p. 114.
59. Morau. quoted by Frank, ibid. p.88.
60. Nibler. Miss. Agri. Stat. Res. Bull.
1929. 150.
- 61.a. Parkes, A.S. The Internal Secretions of the
Ovary, p. 44.
- b. ibid. pp. 45-46.
- c. ibid. pp. 46-47.
- d. ibid. pp. 102-103.
- e. ibid. pp. 105-106.
- f. ibid. p. 160.
- g. ibid. pp. 163-164.

62. Parkes, A.S. and Bellerby, C.W. The significance of the occurrence of oestrin in the placenta. 1927. J. Physiol. 62, 385.
63. Parkes, A.S. and Bellerby, C.W. Studies in the internal secretions of the ovary. V. The oestrus inhibiting function of the corpus luteum. 1927. J. Physiol. 64.
64. Retterer and Konigstein. quoted by Frank, The Female Sex Hormone, p. 88.
65. a. Schafer, E. The Endocrine Organs, Part II, pp. 384-7. 1926.
b. pp. 387-8.
66. Schickel, quoted by Frank, The Female Sex Hormone, p. 112.
67. Seaborn, E. and Champy, Ch. Structure de l'ovaire de la jument et son cycle evolutif en dehors de la gestation. 1923. C.R. Soc. Biol. Paris, 89, 1091.
68. Seaborn, E. The oestrous cycle of the mare and some associated phenomena. 1925. Anat. Rec. 30, 277.
69. Steinach, quoted in The Endocrine Organs, p. 389.
70. Simmonet, H. The properties of the follicular fluid. 1. The concentration and properties of folliculine. 1927. Ann. phys. physicochem. biol. 3, 586.

- 71.a. Smith, F. Veterinary Physiology, 1921.
p. 765-7.
- b. p. 768.
72. Smith, M.G. quoted in The Internal
Secretions of the Ovary, p.108.
73. Smith, P.E. Ablation and transplantation
of the hypothesis in the rat.
1926. Anat. Rec. 32, 221. X
74. Sonnenberg, quoted in The Internal
Secretions of the Ovary, p.84.
75. Stockard and Papanicolaou. The existence of a typical
oestrous cycle in the guinea
pig with a study of its
histological and physiological
changes. 1917. Amer. J. Anat.
22.
76. Turner, Frank A study of the oestrus-producing
Lomas and Nibler. hormone in the urine of cattle
during pregnancy. Miss. Agri.
Stat. Bull. 150.
77. Trivino, F.G. Enlargement of the uterus (in
mice) by administering serum
from pregnant women. 1926.
Klin. Wschr. 5, 2022.
78. Veler, C.D. and Extraction of ovarian hormone
Doisy, E.A. from urine. 1928, Proc. Soc.
Exp. Biol. Med. 25, 806.
79. Wiesner, B.P. Die Phasen des Sexual Zyklus.
1926. Biol. Gen. 2, 1/2.
80. Idem. Die Phasennatur des Sexual
Zyklus. 1927. Arch. Frauen-
kunde, 13.

81. Wiesner, B.P. and Crew, F.A.E. The gonadotrophic actions of the anterior lobe of the pituitary. 1929-30. Proc. Roy. Soc. Edin. 50, 79.
82. Zondek, B. and Aschheim, S. Hypophysen vorderlappenhormon und ovarialhormone in Harn von Schwangeren. 1927. Klin. Wschr. 6, 1322.
83. Idem. Hypophysen vorderlappenhormon und ovarium. 1927. Arch. Gynak. 130, 1.
84. Zondek, B. Darstellung des Weiblichen Sexualhormon aus dem Harn von Schwangeren. 1928. Klin. Wschr. 7, 485.
85. Idem. Schwangerschaftsreaction aus dem Harn bei Mensch und Tierz. 1930. Klin. Wschr. 9, 2285.

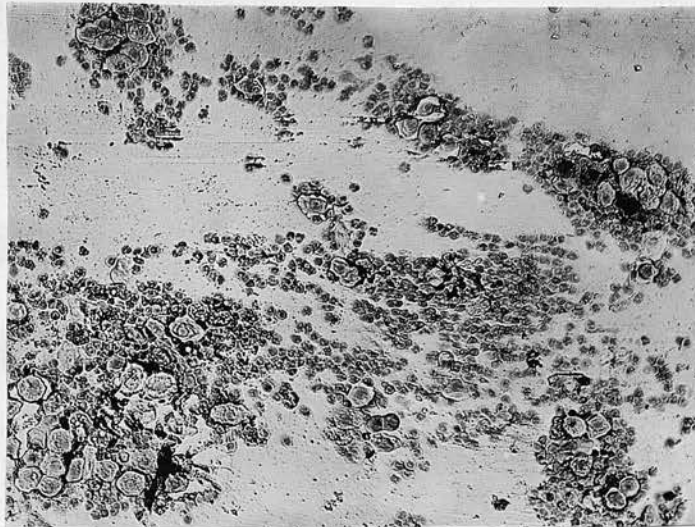


Plate 1. Photomicrograph of vaginal smear taken from mouse which had been injected with urine from a non-pregnant mare. The smear shows leucocytes and nucleated epithelial cells. This represents the stage of dioestrus.

x 110, stained Giemsa.

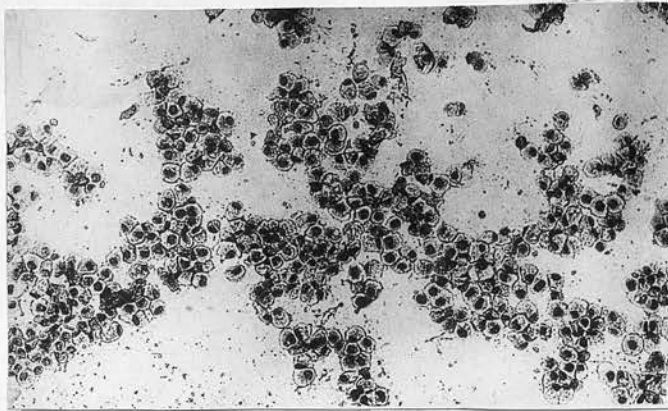


Plate II. Photomicrograph of vaginal smear taken from a mouse which had been injected with urine from a pregnant mare. Nucleated epithelial cells are shown. This represents the stage of pro-oestrus. While this indicates the presence of a certain amount of the alpha hormone, it is not regarded as a positive result.

x 110, stained Giemsa.

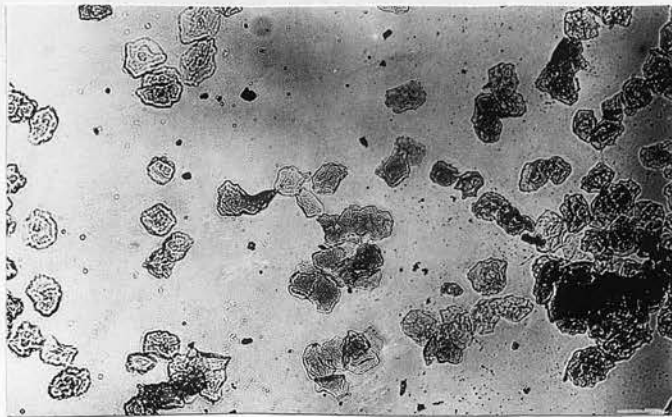


Plate III. Vaginal smear from mouse which had been injected with urine from a pregnant mare. The epithelial cells are completely cornified and leucocytes are absent. This is the stage of oestrus in the mouse and is regarded as positive evidence of pregnancy.

x 110, stained Giemsa.

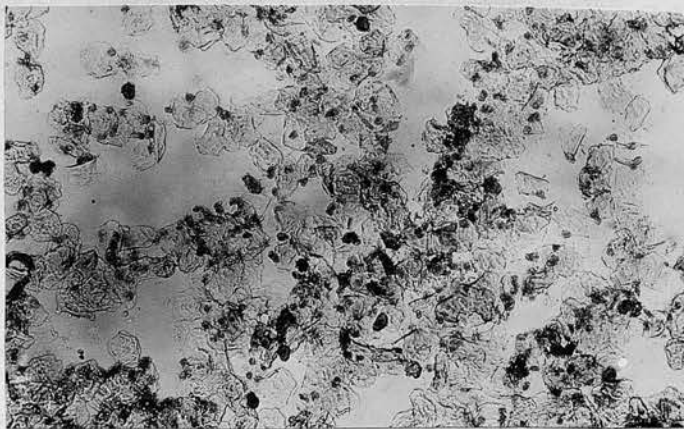


Plate IV. Vaginal smear from the same mouse as in
Plate III. Leucocytes and cornified epithelial
cells are shown. This represents the stage of
metoestrus. After this the vaginal smears grad-
ually return to the condition shown in Plate I.

x 110, stained Giemsa.

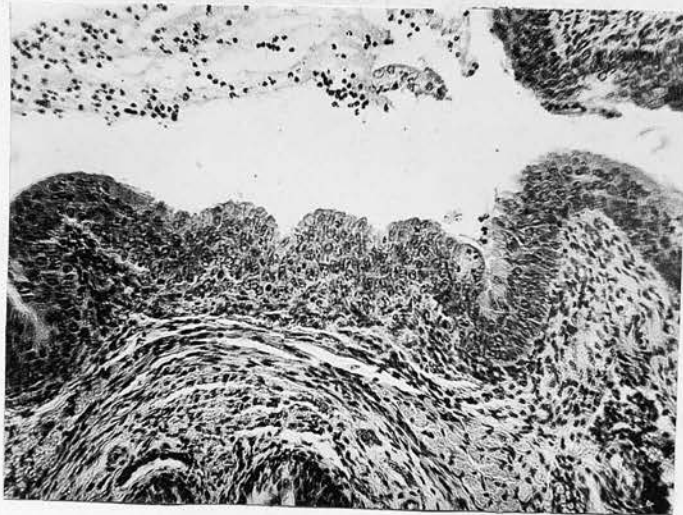


Plate V. Photomicrograph of section of vagina of mouse which had been injected with urine from a non-pregnant mare. The epithelium is shown in its normal resting condition.

x 100, stained H.E.



Plate VI. Section of vagina of mouse which had been injected with urine from a pregnant mare. The mouse was killed on the 5th day of the test. The epithelium has undergone marked proliferation, the surface layers have become keratinised and the lumen is full of cast-off cells. This represents the stage of oestrus.

x 100, stained H.E.

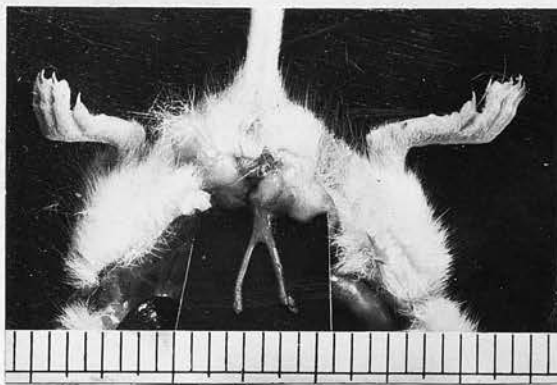


Plate VII. Photograph of the genital tract of an ovariectomised mouse which had been injected with urine from a non-pregnant mare. The uterus is small and undistended as in the dioestral condition.

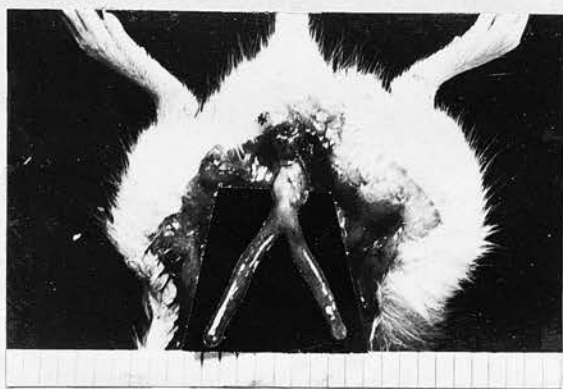


Plate VIII. Genital tract of an ovariectomised mouse which had been injected with urine from a pregnant mare. Note the marked distension of the uterus.